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The Electrophysiological Effects of Acute Thyroid Hormone Exposure on Atrial Myocytes

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THE ELECTROPHYSIOLOGICAL EFFECTS OF ACUTE THYROID HORMONE EXPOSURE ON ATRIAL MYOCYTES

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CHAPTER I

INTRODUCTION

Clinically patients who are chronically hyperthyroid experience an increased heart rate, stroke volume, systolic blood pressure, cardiac output and cardiac contractility along with various atrial irregularities including atrial fibrillation (Klein & Ojamaa, 1994; Ridgeway, 1994; Natazawa *et al.*, 1994). Most of the laboratory research to date has been conducted on ventricular tissue investigating the chronic effects of thyroid hormone exposure, that are mediated by nuclear receptors, leading to alterations in specific gene expression (Polikar *et al.*, 1993; Sap *et al.*, 1986; Franklyn & Gammage, 1996; Oppenheimer *et al.*, Samuels & Tsai, 1973). Various researchers have documented an increase in cardiac contractility (Buccino *et al.*, 1967; Howitt *et al.*, 1968) and a decrease in action potential duration (Freedberg *et al.*, 1970) due to alterations in ion channel properties and contractile protein mRNA synthesis/degradation rates (Johnson *et al.*, 1973; Sharp *et al.*, 1985; Franklyn & Gammage, 1996; Meo *et al.*, 1994; Binah *et al.*, 1987; Meo *et al.*, 1995; Ojamaa *et al.*, 1992). The slow inward current (I_{si}) (Rubinstein & Binah, 1989), the outward rectifying current (I_K) (Rubinstein & Binah, 1989) and the Ca^{2+} current (I_{Ca}) (Binah *et al.*, 1987) all increased with chronic T_3 exposure. Cardiac contractile proteins (α- & β- MHC) mRNA levels as well as associated excitation-

contraction regulatory proteins (SR Ca^{2+}-ATPase, phospholambam, and ryanodine receptors) mRNA synthesis/degradation rates varied with chronic T_3 exposure. While β-MHC mRNA decreased with chronic T_3 exposure (Franklyn & Gammage, 1996; Balkman *et al.*, 1992), α-MHC mRNA increased (Ojamaa *et al.*, 1992; Franklyn & Gammage, 1996; Balkman *et al.*, 1992). SR Ca^{2+}-ATPase (Ojamaa *et al.*, 1992; Arai *et al.*, 1991) and ryanodine receptor mRNA increased (Arai *et al.*, 1991) whereas phospholambam mRNA decreased (Seitz *et al.*, 1994) with chronic T_3 exposure leading to faster cellular contraction rate and an increased rate of cellular relaxation due to increased Ca^{2+} SR re-uptake.

Only recently have the acute effects of thyroid hormone exposure been investigated. Initially there was controversy about whether the effects seen after a brief bout of T_3 exposure were in fact due to different mechanisms from the nuclear mediated effects. Now there is documented proof of plasma membrane T_3 receptors (Segal, 1989). Previously there has been documented increases in the Na^+ current in neonatal cardiac myocytes (Harris *et al.*, 1991) and rat ventricular myocytes (Dudley & Baumgarten, 1993) and increases in the cellular Ca^{2+} uptake in rat heart slices (Segal, 1990) and the isolated perfused heart (Gøtzsche, 1994) with acute T_3 exposure. Little is known, however, about the acute effects of T_3 on atrial muscle cells from the adult heart. It is not known whether the previously documented chronic effects altering cardiac contractility and conduction irregularities are attributable to changes seen during an acute exposure to T_3. With previous documented effects of acute T_3 exposure affecting Na^+ current (Harris *et al.*,

1991; Dudley & Baumgarten, 1993) and cellular Ca^{2+} uptake (Segal, 1990; Gøtzsche, 1994), the present research was conducted to investigate the acute effects of thyroid hormone on atrial muscle cells. These experiments will investigate whether the acute effects of T_3 exposure are similar to the previously documented chronic effects on cardiac function. Results from this research will provide a greater understanding of the cellular mechanisms present during hyperthyroidism and lead to an understanding of potential arrhythmogenic and therapeutic effects of acute thyroid hormone replacement.

CHAPTER II

LITERATURE REVIEW

A. Synthesis of Thyroid Hormone

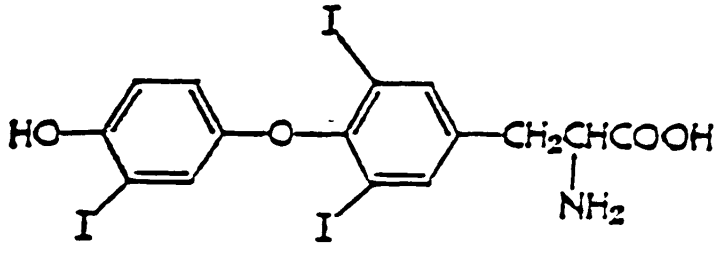
T_4/T_3 production in the thyroid gland. The thyroid gland is the source of two major hormones, tetraiodothyronine (T_4) and triiodothyronine (T_3). T_3 is considered the biologically active thyroid hormone due to its 10 fold higher affinity for the nuclear thyroid hormone receptor (Berne & Levy, 1993). The synthesis of thyroid hormone occurs within the thyroid gland on thyroglobulin. Within the follicle, thyroglobulin is iodinated to yield both monoiodotyrosine (MIT) and diiodotyrosine (DIT) with coupling of mono and diiodotyrosine moieties to form T_4 (DIT + DIT) or T_3 (DIT+ MIT) (Genuth, 1993). Secreted from the thyroid gland, thyroxine (T_4) constitutes approximately 85-90% of the output with T_3 comprising about 10-15 % and reverse T_3 (rT_3) accounting for only 1 % (Sypniewski, 1993; Genuth, 1993). The half lives of T_4 and T_3 are 7 and 1.5 days respectively (Sypniewski, 1993). The majority of T_3 available to the body tissue is derived outside of the thyroid gland from the peripheral conversion of T_4 to T_3 by the 5'-monodeiodinase enzyme located primarily in the liver and kidney (Astwood, 1970).

Cellular thyroid hormone uptake. Once released into the blood stream, T_4 and T_3 are primarily bound to the blood proteins thyroid-binding globulin, thyroid-binding

Figure 1

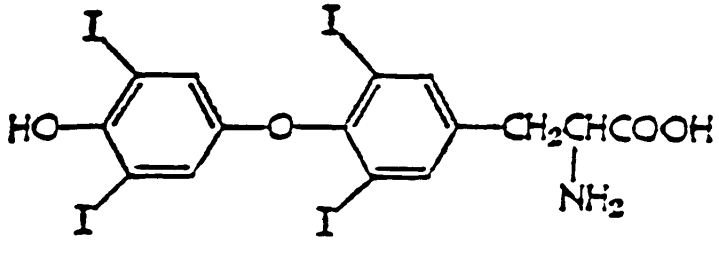
Thyroid Hormone Chemical Structures

A



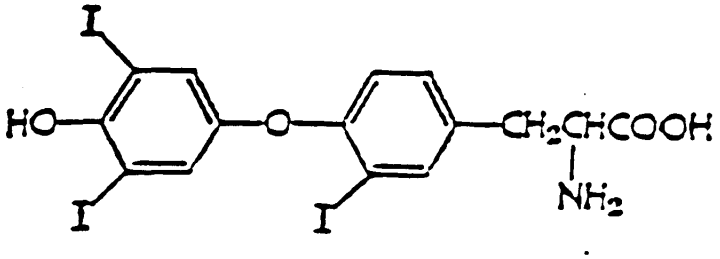
T_3 (3,5,3'-Triiodo-L-thyronine)

B



T_4 (L-Thyroxine)

C



rT3 (3,3',5'-Triiodo-L-thyronine)

Thyroid hormone chemical structures.
(modified from Goodman & Gilman, 1970)

prealbumin, and albumin with only 0.02-0.03% and 0.2-0.3% respectively representing the biologically active free form (Sypniewski, 1993; Genuth, 1993; Astwood, 1970). Two theories of cellular thyroid hormone uptake have been presented. Originally, Lein and Dowben (1991) offered indications that triiodothyronine uptake into the tissue occurred as simple diffusion, which seemed to correlated well with the lipophilic properties of thyroid hormone. Recent research in thyroid hormone uptake provides evidence that there is a saturable, stereospecific, energy dependent processes which is responsible for T_3 uptake in rat skeletal muscle (Pontecorvi & Robbins, 1986). Oppenheimer & Schwartz (1985) provide further evidence of a stereospecific transport of thyroid hormone into the nucleus due to the preferential accumulation of L-T_3 versus D-T_3 in the intact animal.

Once in contact with the nuclear or plasma membrane receptors, thyroid hormone is able to influence the cell leading to changes in the metabolic, mechanic and electrophysiologic properties.

B. Clinical Effects of Hyperthyroidism

The biological hallmark of abnormal thyroid function is a change in the basal metabolic rate (Klein, 1990). Thyroid hormone acts primarily to stimulate cellular oxygen consumption and substrate utilization (Muller *et al.*, 1994). Hyperthyroidism is characterized by elevated serum levels of T_3 and T_4, due to either an overproduction of T_3 and T_4 in the thyroid gland, an increased release of thyroid hormone from the thyroid gland or an exogenous administration of thyroid hormone. The pharmacological actions

of thyroid hormone include alterations in cardiovascular function and in metabolism of glucose, fatty acid and protein. Hyperthyroidism has been documented to produce a positive cardiac inotropic as well as chronotropic effect with potential arrhythmogenic effects, such as atrial fibrillation (Ridgeway, 1994; Ito *et al.*, 1994). Cardiovascular actions of T_3 can be attributed to (1) direct effects upon the heart, (2) effects upon the peripheral circulatory system, or (3) interactions with the sympathetic/adrenergic system (Nicod *et al.*, 1993). Cardiovascular manifestations of a sustained elevation in thyroid hormone levels include an increased heart rate, myocardial contractility, stroke volume, systolic blood pressure, cardiac output, a widened pulse pressure and a decreased diastolic blood pressure (Klein & Ojamaa, 1994). Contributing to these cardiovascular affects of T_3 are alterations in ion channel properties and associated contractile protein synthesis/degradation rates.

Mechanic. Mechanically, when the heart is placed under a hyperthyroid condition contractility increases. Buccino *et al.* (1967), using cat papillary muscles, found a more rapid rate of tension development with less time to reach peak tension in the muscles from chronic hyperthyroid animals, though the maximum tension developed was not significantly greater than from euthyroid animals. Howitt *et al.* (1968), reported indirect indices of increased contractility in humans by showing a shorter ejection time and increased mean systolic ejection rate, suggesting that ejection velocity was enhanced.

Electrophysiologic. Hyperthyroidism effects the electrophysiology of the heart by altering various ionic currents which underlie the generation of the action potential at

the single cell level, effecting the conduction and eventual contractile behavior of the heart organ. Tachycardia associated with hyperthyroidism has been shown to be due to a combination of a decrease in action potential duration coupled with an increased rate of diastolic depolarization in the sinoatrial node pacemaker cells (Marshall, 1973). Freedberg *et al.* (1970), using isolated atria from rabbits, showed that although the resting membrane potential and the height of the action potential did not differ, the duration of the action potential repolarization was shortened in animals who received T_4 for 6 weeks. In hyperthyroid dogs, A-V functional refractory period and conduction time were both significantly shortened as the A-H interval decreased with no change in H-V interval (Goel, 1972).

Hemodynamic. Hemodynamically, hyperthyroidism places altered preload and afterload forces upon the heart muscles. Thyroid hormone has been shown to lead to peripheral vasculature relaxation (Klein *et al.*, 1994; Dillman, 1989) with decreased circulation time (Smallridge, 1980). This relaxation was due to the direct effect of thyroid hormone on vascular smooth muscle, without influence from endothelium-derived relaxing factor(s), α- or β- receptor activation, or muscarinic cholinergic receptor activation (Hidaka *et al.*, 1989). An increased blood volume (Klein, 1994), cardiac filling (Dillman, 1994), stroke volume, heart rate, coronary blood flow and oxygen consumption were also present during hyperthyroidism (Smallridge, 1980).

Metabolic. Sustained elevated thyroid hormone levels have been shown to effect intermediary metabolism, stimulating oxygen consumption and oxidation of glucose, fatty

acids and amino acids. Mitochondrial adenine nucleotide translocase proteins and respiratory chain enzymes increased under hyperthyroid conditions in the liver. Glucose metabolism increased with thyroid hormone due to an increased amino acid uptake by the liver, an increased activity of gluconeogenic enzymes and an increased oxygen consumption leading to an increase in ATP supply for many metabolic pathways including glucose production. In liver, fatty acid oxidation (β-oxidation) and ketone body production increased with thyroid hormone. Hyperthyroidism is usually accompanied by a negative nitrogen balance with muscle wasting being an obvious sign but surprisingly heart muscle protein did not decrease but actually increased, due to the multiple factors placed upon the heart muscle under elevated thyroid hormone levels (Seitz *et al.*, 1994).

Due to the clinical manifestations of hyperthyroidism, such as tachycardia, nervousness, and tremors, mimicking a hyperadrenergic state, researchers have investigated whether the response to thyroid hormone resulted from a stimulation of the sympathetic nervous system (Polikar *et al.*, 1993). Plasma levels of catecholamines were normal or low in hyperthyroidism (Coulombe *et al.*, 1976). Williams *et al.* (1977) reported in rats that chronic administration of thyroid hormone up regulated the number of β-adrenergic receptors leading to an increased cardiac catecholamine sensitivity. Therefore the hyperadrenergic state seen in hyperthyroidism suggest a hypersensitivity to catecholamines rather than an increase in sympathetic activity, because plasma catecholamine levels are normal or decreased during hyperthyroidism.

C. Cellular Effects of Hyperthyroidism-Chronic

Nuclear Receptor Binding. At the cellular level, it is well recognized that the biologically active thyroid hormone (T_3) exerts its action by binding to specific thyroid hormone receptors, a protein product of the cellular erythroblastosis A (*c-erb* A) supergene family, located on the nuclear membrane (Franklyn & Gammage, 1996; Polikar *et al.*, 1993; Sap *et al.*, 1986; Oppenheimer *et al.*, 1972; Samuels & Tsai, 1973). Researchers have discovered binding sites for thyroid hormone on nuclei isolated from atrial and ventricular cardiac muscle (Banerjee *et al.*, 1988), and on liver and kidney tissue (Oppenheimer *et al.*, 1972). Upon thyroid hormone binding to its nuclear receptor with subsequent receptor binding to various enhancer/promoter sequences on DNA, gene transcription is stimulated or inhibited (Franklyn & Gammage, 1996; Polikar *et al.*, 1993; Sap *et al.*, 1986). Changes in the quantity or properties of such proteins that are important in the modulation of cardiac contraction may underlie the contractile changes that accompany hyperthyroidism.

Contractile Associated Proteins. It has been shown in rat heart that chronic T_3 exposure induces synthesis of α-myosin heavy chain (MHC) mRNA while exerting an inhibitory effect on β-MHC synthesis (Franklyn & Gammage, 1996), leading to an increase in myosin V_1 and a decrease in myosin V_3 isoenzymes (Dillman, 1990). In a time course experiment, Balkman *et al.* (1992) showed that hypothyroid rats increased α-MHC mRNA over time, eventually reaching 93% ± 1% of total MHC mRNA, coupled with a decrease in the β-MHC mRNA levels. Myosin V_1, composed of 2 MHC α

subunits, has a higher myosin ATPase activity than myosin V_3, which contains 2 MHC β subunits. The globular head of myosin V_1 with its higher myosin ATPase activity than myosin V_3 resulted in an increased velocity of contraction (Dillman, 1990). This marked thyroid hormone-induced change in myosin isoenzyme predominance was demonstrated in small animals (rats, rabbits) but did not occur to a similar extent in larger species, including the human heart in which V_3 is the major ventricular isoenzyme (Franklyn & Gammage, 1996).

In rabbit skeletal and cardiac muscle chronic T_3 administration increased the steady state levels of cardiac ryanodine receptor mRNA and SR Ca^{2+}-ATPase mRNA when compared with euthyroid rabbit (Arai *et al.*, 1991). The SR Ca^{2+}-ATPase functions to regulate the intracellular calcium concentration of the myocyte and therefore affects the rate of Ca^{2+} release and uptake, affecting the rate of systolic tension development and the diastolic relaxation time (Arai *et al.*, 1991). Ojamaa *et al.* (1992) demonstrated in a heterotropic isografted rat heart that although thyroid hormone required an increased hemodynamic load to increase cardiac protein synthesis, thyroid hormone altered the expression of α-MHC and SR Ca^{2+}-ATPase mRNA concentration, increasing them 181 and 208% versus euthyroid animals respectively. SR Ca^{2+}-ATPase mRNA which had been depressed in hypothyroid when compared with euthyroid rat hearts, responded to T_4 with elevated SR Ca^{2+}-ATPase mRNA levels reaching 255% of control after 72 hours (Balkman *et al.*, 1992).

Phospholambam, a SR Ca^{2+}-ATPase regulatory protein, mRNA was decreased in

both hyperthyroidism and hypothyroidism while calsequestrin mRNA levels exhibited no change with thyroid status, indicating that thyroid hormone does not alter the calcium storage capacity to the SR (Seitz *et al.*, 1994). T_3 also has been shown to increase production of mRNA encoding for Na-K ATPase, which is responsible for restoring the Na/K gradients and membrane potential after excitation (Evert, 1996).

In a developmental study, rat ventricular myocyte TnI mRNA levels were higher in euthyroid versus hypothyroid. Upon addition of T_3 to young rats (<28 days) TnI mRNA increased versus control, but the effect was not seen after 87 days of gestation, suggesting that the level of TnI mRNA was more sensitive to thyroid status in younger rats (MacKinnon & Morgan, 1986). Other studies provide further evidence for specific mRNAs being induced by T_3 in a highly tissue specific manner (Shanker *et al.*, 1987).

Ionic Currents. Chronic T_3 exposure also effects various ionic currents in cardiac and skeletal muscle. Hyperthyroidism increased the slow inward current (I_{si}), shifting the threshold for activation as well as the peak current to more negative potentials when compared with control, while hypothyroidism decreased the I_{si} current (Rubinstein & Binah, 1989). The outward rectifying current (I_K) increased with chronic T_3 exposure but hypothyroidism had little effect on I_K (Rubinstein & Binah, 1989). The peak calcium current also increased in hyperthyroid ventricular myocytes (Binah *et al.*, 1987). Hypothyroidism, in rat ventricular myocytes, decreased the transient outward potassium current (I_{ss}) while hyperthyroidism increased the amplitude of I_{ss} (Shimoni & Severson, 1996).

Cardiac Inotropic Response. Incorporating the ionic current and contractile protein alterations with T_3, the effect of thyroid hormone on cardiac function has been demonstrated on action potential and cardiac contractile mechanics. T_3 exhibited positive inotropic characteristics leading to a decrease in action potential duration (Johnson *et al.*, 1973; Meo *et al.*, 1994;Binah *et al.*, 1987; Meo *et al.*, 1995) and a decrease in the time to peak tension development (Sharp *et al.*, 1985). In rat ventricular myocytes, heart rate increased as well as the ventricle displayed a faster rate of contraction and relaxation leading to a shortened contraction cycle when compared with euthyroid myocytes. There was no change in maximum tension developed by the ventricle, but the duration of the contraction was shorter. This study concurrently measured the calcium transient which was shorter in duration with T_3 and the rise and decline of the calcium transient which exhibited faster kinetics than in euthyroid cells (MacKinnon & Morgan, 1986).

Vascular Smooth Muscle. Thyroid hormone directly effected vascular smooth muscle, leading to vascular relaxation in the presence of the β-receptor blocking drug propranolol, the α-receptor blocker phentolamine and the muscarinic cholinergic blocking agent atropine. Endothelium-derived relaxing factor(s) could also be ruled out as the mechanism of thyroid hormone induced vascular relaxation as thyroid hormone displayed its effect on tissue in which the endothelium was intact or removed (Ishikawa *et al.*, 1989).

D. Cellular Effects of Hyperthyroidism- Acute

Plasma Membrane Mediated Actions. In the last couple of decades the acute effects of thyroid hormone on skeletal, smooth and cardiac muscle have been investigated. Focus has turned to the possible plasma membrane-mediated effects of thyroid hormone, apart from the documented nuclear membrane-mediated effects seen with chronic thyroid hormone exposure. Characteristically, the plasma membrane-mediated effects of thyroid hormone are prompt in onset (seconds to minutes), independent of new protein synthesis, and are associated with changes in the transmembrane transport of ions and substrates (Segal, 1989). Extranuclear sarcolemmal effects of thyroid hormone include effects on the inward rectifier potassium current (Sakaguchi *et al.*, 1996), the sodium current (Harris *et al.*, 1991; Dudley & Baumgarten, 1993), sugar (2-deoxyglucose) transport (Segal & Ingbar, 1990; Segal, 1989), Ca^{2+}-ATPase (calcium pump) activity (Segal *et al.*, 1989; Davis *et al.*, 1983), and calcium release from ryanodine receptors (Connelly *et al.*, 1994). Also shown during acute thyroid hormone infusion has been an effect on cardiac conduction time (Gøtzsche, 1994), contractility (Gøtzsche, 1994; Ririe *et al.*, 1995), and cardiac output (Gøtzsche, 1994). In addition to the plasma membrane mediated actions of acute thyroid hormone exposure, thyroid hormone has been shown to promote extranuclear effects on some cytosol proteins (protein kinases) (Lawerence *et al.*, 1989) and on SR Ca^{2+}-ATPase activity (Warnick *et al.*, 1988).

Ionic Currents. Ionic currents that modulate various cardiac functions have been investigated during acute thyroid hormone exposure. In ventricular guinea pig myocytes,

Sakaguchi *et al.* (1996) demonstrated an increase in the inward rectifier potassium current (I_{K1}) with 1 nM T_3. At the single channel level thyroid hormone application increased the mean open time (P_o) of the inward rectifier potassium channel (I_{K1}) due mainly to a decreased interburst duration, with no change in the single channel amplitude or slope conductance. It is known that I_{K1} is the main contributor to the late phase of action potential repolarization (Ibarra *et al.*, 1991). Therefore, these researchers suggested that thyroid hormone may shorten the action potential duration by enhancement of I_{K1} (Sakaguchi *et al.*, 1996).

Dudley and Baumgarten (1993) have investigated the effects of acute exposure of thyroid hormone on cardiac sodium channels in rat ventricular myocytes, showing that T_3 (5 or 50 nM) induced an increase in the bursting behavior of the channel without a change in the unitary conductance. Using TAA, which does not bind to nuclear thyroid hormone receptors (De Nayer, 1989), bursting behavior increased similarly to T_3, indicating that with in the experimental time frame T_3 was not mediating its effects through nuclear receptors. They further reported that T_3 only increased channel bursting behavior when applied to the extracellular side of the membrane (Dudley & Baumgarten, 1993).

In neonatal cardiac myocytes, thyroid hormone (5-20 nM) application doubled the peak of the inward sodium current with 2 minutes, eventually plateauing at 4 fold control levels after 4 minutes of hormone application. Sodium current inactivation shifted from a monoexponential decay to a bi-exponential fit in the same time period. Steady state

inactivation of sodium current, though, was unaffected by T_3 (Harris *et al.*, 1991).

Ca^{2+} & Glucose Uptake. In rat heart slices, the effect of T_3 (10 pM) on calcium and glucose uptake was investigated. Thyroid hormone acutely increased calcium (^{45}Ca) uptake that was blocked with inorganic calcium channel blockers (La^{3+}, Cd^{2+}, and Mn^{2+}) (Segal, 1990) and increased 2-deoxyglucose uptake, in the presence of cyclohexamide demonstrating that the effects were independent of new protein synthesis (Segal, 1989). Segal (1990) further demonstrated that the molecular mechanism of action of thyroid hormone was mediated by calcium as the first messenger for the acute plasma membrane mediated effects of sugar uptake. In a whole animal experiment, Gøtzsche (1994) showed that acute T_3 administration to a rat resulted in a significant, reversible decrease in calcium perfusate concentration, which was blocked with the calcium channel blocker, nifedipine, indicating that the thyroid hormone induced calcium flux into the cell might be partly mediated through voltage dependent calcium channels.

SR Ca^{2+}-ATPase Activity. Thyroid hormone has been found to regulate sarcoplasmic reticulum membrane Ca^{2+}-ATPase activity by nonnuclear mechanisms. SR Ca^{2+}-ATPase activity determines the rate of calcium reuptake and hence the rate at which myocardial relaxation occurs. In striated muscle, SR Ca^{2+}-ATPase activity increased with T_3 exposure (Warnick *et al.*, 1988).

Calcium Release Channels. Thyroid hormone (250 mM) has also been shown to increase activation of calcium release channels (ryanodine receptor) in skeletal muscle SR at the single channel level in planar lipid bilayers (Connelly *et al.*, 1994). The open

probability (P_o) increased significantly in the presence of thyroid hormone. These researchers demonstrated that T_3 produces its effect through a direct activation of the ryanodine receptor in the absence of other cellular components.

β-adrenergic Receptors. Because the clinical signs of hyperthyroidism mimic a hyperadrenergic state, the acute effects of thyroid hormone on β-adrenergic receptors were investigated in cultured chick cardiac myocytes. Initially (under 2 hours), T_3 (10 nM) application resulted in an increased number of receptors on the cell membrane, which was not influenced by the protein synthesis inhibitor cyclohexamide (20 mM). When colchicine (20 mM) was added to the cultured cells, T_3 did not lead to an increase in β-receptor number indicating that the effects of thyroid hormone initially did not depend upon new protein synthesis but relied on microtubule assembly to promote insertion of already synthesized receptors into the plasma membrane from the cytosol (Vassey *et al.*, 1994).

cAMP levels. Levey and Epstein (1968) demonstrated in cat heart homogenates that thyroid hormone ($5x10^{-6}M$) activated adenyl cyclase leading to cAMP accumulation at levels significantly higher than control animals within 3 minutes. This cAMP accumulation was independent of diminished degradation by phosphodiesterase indicating a direct effect by thyroid hormones on regulating activation of adenyl cyclase. Also since the effect was produced within 3 minutes, it would suggest that thyroid hormone increased activity due to actual activation of adenyl cyclase rather than generation of new enzymes because the time frame appears to be too short for new protein synthesis to

occur.

Intracardiac Conduction Time. Shahawy *et al.* (1975) demonstrated that acute thyroid hormone exposure in dogs resulted in a decrease in the intracardiac conduction time. During thyroid hormone perfusion the heart rate increased, the A-H interval decreased while the H-V interval remained constant when compared with euthyroid animals. In triiodothyronine treated pigs, cardiac contractility increased significantly after 15 minutes (Gøtzsche, 1994). Cardiac output increased along with contractile force ($^{+}$dP/dt) which was blocked with amiodarone suggesting the mechanism of action of thyroid hormone was by altering the permeability properties of the calcium channel on the sarcolemmal membrane (Gøtzsche, 1994).

Cardiac Contractility. In an isolated rat heart, Ririe *et al.* (1995) demonstrated that a bolus injection of T_3 (0.74 nM) was able to increase left ventricle contractility ($^{+}$dP/dt) within 30 seconds with no change in end diastolic pressure. T_4 was unable to increase contractility even at levels 17 times (12.9 nM) the bolus injection of T_3 indicating a specificity for T_3 in mediating the effects of acute hyperthyroidism. The effects of T_3 were transient, with the increased contractility dissipating over a period of 60-90 seconds after drug administration. β-receptor blockade with propranolol (1 mM) did not effect the ability of T_3 to increase contractility, as maximum developed tension with and without β-receptor blockade was 393 ± 29 and 335 ± 38 mmHg/sec, respectively. T_3, at concentrations that had been shown to increase contractility and even as high as 10^{-6} M, exhibited no effect on cAMP production (Ririe *et al.*, 1995), in contrast to the earlier work

done by Levey and Epstein (1968). At the single cell level, in isolated ventricular myocytes, T_3 caused a dose dependent increase in contractility independent of β-receptor stimulation (Walker *et al.*, 1994).

Vascular Smooth Muscle. Hyperthyroidism has been shown to decrease systemic vascular resistance. Since vascular smooth muscle tonic contraction is a major determinant of vascular resistance (Berne & Levy, 1993), the acute effects of thyroid hormone on smooth muscle have been investigated. Thyroid hormone (10-30 mM) relaxed isolated rabbit mesenteric artery smooth muscle strips previously contracted with 50 mM KCl. This relaxation was due to the direct effect of thyroid hormone on vascular smooth muscle, without influence from endothelium-derived relaxing factor(s), α- or β-receptor activation, or muscarinic cholinergic receptor activation (Ishikawa *et al.*, 1989). In cultured smooth muscle cells, addition of T_3 (0.1 nM) produced a visible relaxation of the cells in 10 minutes (Ojamaa *et al.*, 1994).

CHAPTER III

METHODS

A. Single Cell Isolation Procedure

Adult cats of either sex, weighing approximately 2 kg were anesthetized with sodium pentabarbitol (80 mg/kg; IP). Single atrial muscle cells were isolated according to methods previously reported (Wu *et al.*, 1991; Zhou & Lipsius, 1992,93a,b). To remove the heart a midsternal thoractomy was performed. The heart was quickly attached to a Langendorff perfusion apparatus at the aorta. The heart was perfused for 5 minutes with a Tyrode solution at 37°C until the blood was washed out completely (Table 1, Solution 1). This was followed by a second perfusion of 5 minutes with a Ca^{2+}-free Tyrode solution (Table 1, Solution 2) and a final perfusion of 50-60 minutes with a Tyrode solution containing 36 μM Ca^{2+}, 0.1% albumin, and 0.06% collagenase (Table 1, Solution 3). The enzyme solution was recirculated with a pump. During the enzyme recirculation period, every 10 minutes 27 μM Ca^{2+} was added to the perfusate to eventually yield a Ca^{2+} concentration of approximately 200 μM. The two atria were removed from the heart and separated from one another by cutting through the sepal region of the atria. Atrial tissue was cut into small pieces (<0.5 cm^2) and incubated in

fresh enzyme solution for 15 minutes at 37°C while being agitated in a water bath. The tissue suspension was filtered through a nylon mesh (210 μm) using Tyrode solution containing 200 μM Ca^{2+} and 0.1% albumin. After allowing the cells to settle for around 30 minutes, the superfusate was suctioned off and the isolated cells were gradually replaced with and stored at room temperature until use in a HEPES-buffered solution (Table 2, Solution 1).

Cells used for study were transferred to a small tissue bath (0.4 ml) mounted on the stage of an inverted microscope (Nikon Diaphot) and superfused with a modified HEPES Tyrode solution (Table 1, Solution 4). Solution was perfused by gravity at approximately 4 ml/min. Cells selected for study were elongated, exhibited nice striations, and were quiescent.

B. Patch-Clamp Recording Technique

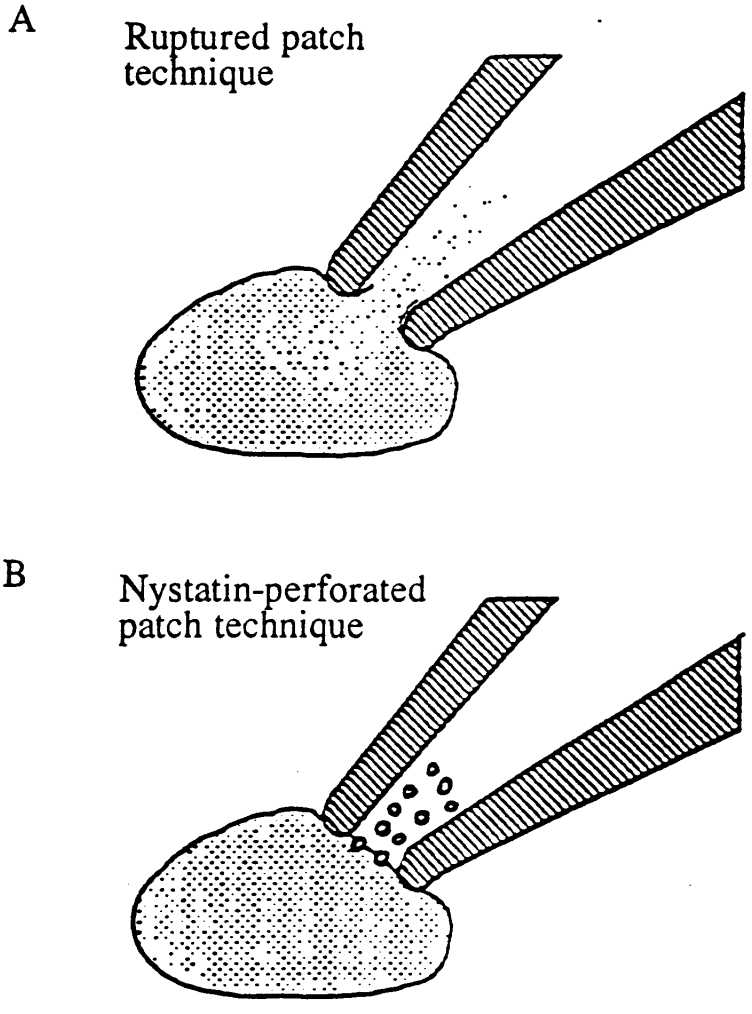
Ruptured patch recording method. Cells used for experiments were transferred to a tissue bath (0.4 ml) mounted on the stage of an inverted microscope (Nikon Diaphot). Sodium currents were measured on atrial muscle cells using the ruptured patch whole cell recording technique (Hamill *et al.*, 1981). Pipettes were pulled from borosilicate glass (Sutter Instrument Co.) in a six step process using a horizontal programmable puller (Flaming Brown, model PC 80, Sutter Instrument Co.) Pipettes were fire polished to smooth out the electrode tip by passing current though a platinum wire in close proximity to the pipette tip. Pipettes were first briefly dipped in the pipette solution to fill the tip

and the pipette was backfilled with the same solution (Table 3, Solution 1). Pipettes had internal diameters of 1.0-1.5 μm and when filled with internal solution yielded a final resistance of 3-4 MΩ when placed in the perfusion bath. Capacitance and pipette resistance were compensated before recording from each cell. Upon touching the cell membrane, negative pressure was applied to form a gigaseal. After a stable gigaseal was obtained (2-5 minutes) additional suction was applied to rupture the membrane patch. Before patch rupture cells were perfused with HEPES Tyrode solution (Table 2, Solution 1). After patch rupture cells were perfuse with a Low Na^+ Tyrode solution (Table 2, Solution 2).

Nystatin-perforated patch recording method. L-type Ca^{2+} currents and action potential recordings were measured on atrial muscle cells using the nystatin perforated-patch whole cell recording technique (Horn & Marty, 1988). Nystatin was dissolved in dimethylsulfoxide at a concentration of 50 mg/ml and then added to the internal pipette solution to yield a final concentration of 150 μg/ml. The nystatin containing pipette solution was strongly sonicated so that the nystatin was completely dispersed in the solution leaving to pipette solution a clear pale yellow. Pipettes were pulled from borosilicate glass by a horizontal programmable puller and fire polished. Pipette internal diameter was 1.0-1.5 μm and when filled with internal solution (Table 3, Solution 2) yielded a final resistance of 2-3 MΩ when placed in the perfusion bath. Capacitance and pipette resistance were compensated before recording from each cell. After the electrode made contact with the cell surface a slight negative pressure was delivered to form a

Figure 2

Cellular Patch Clamp Recording Technique



A. The standard ruptured patch recording technique.
B. The nystatin-perforated patch recording technique.
(modified from Horn & Marty, 1998)

gigaseal between the cell and the electrode. Before patch attachment, cells were perfused with HEPES Tyrode solution. After patch seal was established, a High Cs^+ Tyrode solution (Table 2, Solution 3) was used to perfuse the external cell surface at a temperature of 35°C.

C. Action Potential Recording Method.

Action potentials were elicited by stimulation through the recording electrode in the bridge mode using 2-3 msec voltage pulses. Pulses were generated by an external stimulus (Pulsar 4i Stimulator, Frederick Haer & Co.; Brunswick, Maine) and recorded by computer and magnetic reel to reel tape.

D. Cell Shortening Recording Method.

Video Edge Detector. The cells were superfused with HEPES-Tyrode solution (Table 2. Solution 1) at 35°C. The nystatin perforated-patch technique was used to gain access to the cell. A stimulus generated from an external source was applied to the cell through the cell- attached electrode and the cell shortening produced was measured with the use of a video edge detector with data stored by the computer and magnetic reel to reel tape. The spatial resolution of the video edge detector at 400x magnification is 0.015 microns.

E. Electronic equipment.

Ionic currents (discontinuous single electrode voltage clamp) were recorded in the ruptured patch whole cell configuration and nystatin perforated-patch whole cell method with an Axoclamp-2-A amplifier (Axon Instruments, Inc., Foster City, Calif.). Action potentials were recorded in the bridge mode of the Axoclamp-2-A amplifier. Cells were externally stimulated through the recording electrode by a Pusar 4i Stimulator (Frederick Haer & Co., Brunswick, Maine). Cell shortening was measured with a Video Edge Motion Detector (Cresent Electronics; Sandy, Utah). In the voltage clamp mode the sampling rate was 10-12 kHz. Computer software run on a Dell 386 (PClamp 6.0 program; Axon Instruments, Inc.) was used to generate the voltage clamp protocols as well as record and analyze voltage and ionic current signals. Voltage and ionic currents were sampled by a 12-bit resolution A/D converter (Tecmar Labmaster, Tecmar Inc., Cleveland, OH) with the use of a Dell 386 computer. Data was stored on hard disc and backed up on a bernuilli disk for later analysis.

F. Experimental Voltage Protocols.

Na^+ Current. Currents were measured using the ruptured patch whole cell technique (Hamill *et al.*, 1981). Ionic currents were activated from atrial cells by depolarizing clamp steps from a holding potential of -80 mV in 10 mV increments to +50 mV for 80 msec. Two seconds between incremental clamp steps were used to allow from complete channel recovery from inactivation. Peak Na^+ clamp steps were from holding

potential of -80 mV to -40 mV. Peak Na^+ current was measured with respect to steady state current.

Na^+ Channel Steady State Inactivation. Channel kinetics were measured using the ruptured patch whole cell technique. Cells were held at -120 mV and clamped in 10 mV increments up to +20 mV for 1 second. Following the initial incremental clamp, the cell was returned to -120 mV for 2 msec, after which the cell was clamped to -40 mV. Steady State inactivation for each cell was normalized using the maximum peak current recorded for that particular cell.

Na^+ Channel Steady State Activation. Measured using the ruptured patch whole cell technique. Cells were held at -80 mV and clamped for approximately 2 msec, or a duration not leading to channel inactivation, in 10 mV voltage steps from -80 to +50 mV. After the conditioning prepulse to various voltages, the cell was clamped back to -60 mV and the tail current elicited was measured. Steady state activation was determined by comparing the tail current elicited at each varying prepulse voltage step with the maximum current elicited, yielding a normalized steady state activation curve.

Na^+ Channel Recovery from Inactivation. Measured using the ruptured patch whole cell technique. Cells were held at -110 mV and clamped for a conditioning pulse to -40 mV for 1 second. Following the conditioning pulse was a variable time interval (from 0.2 msec to 150 msec) at -110 mV after which the cell was clamped to -40 mV for 50 msec. The test current was compared with the conditioning current (I_{test}/I_{cond}) at each recovery time to yield a normalized value for each cell.

Figure 3
Experimental Voltage Protocols

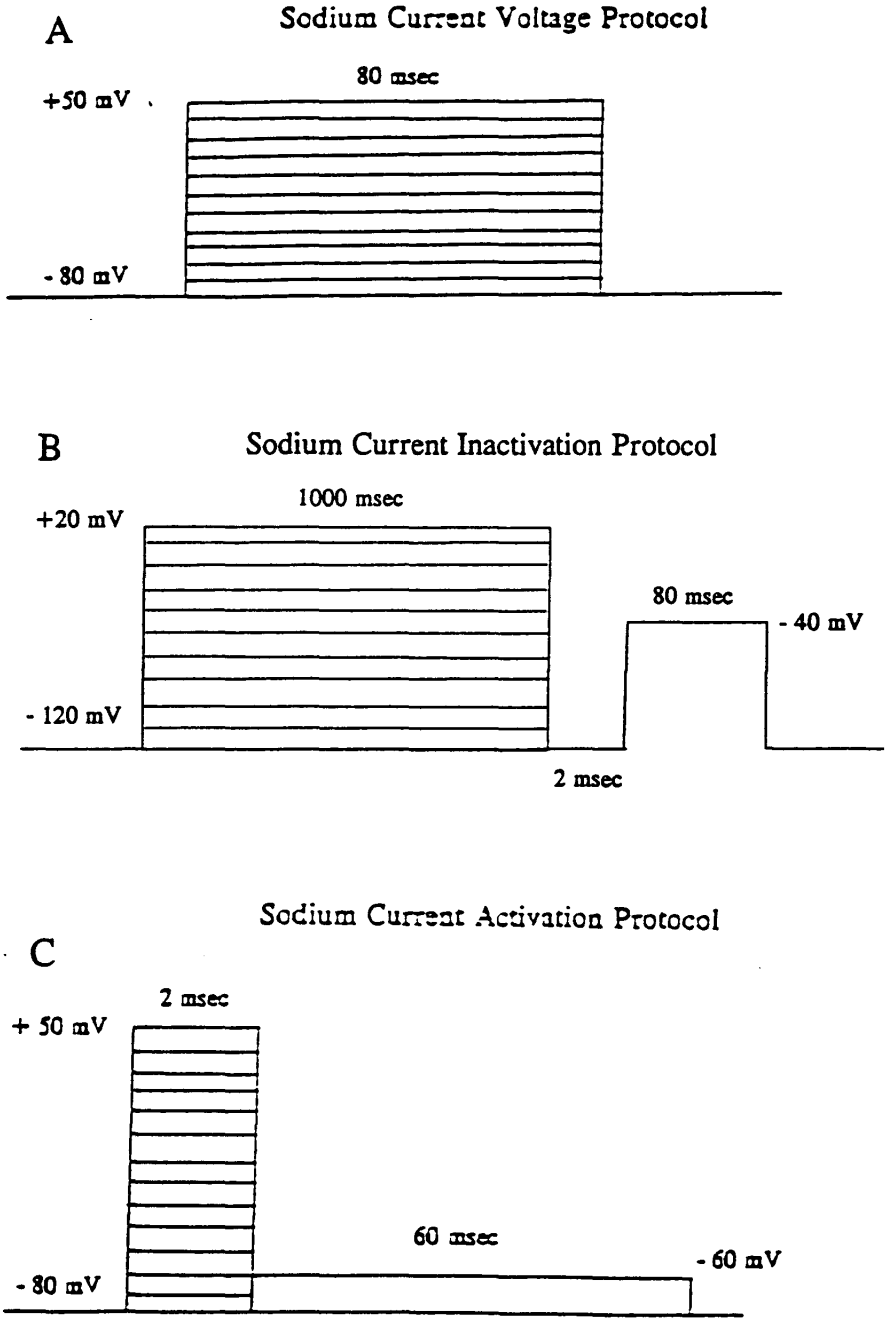
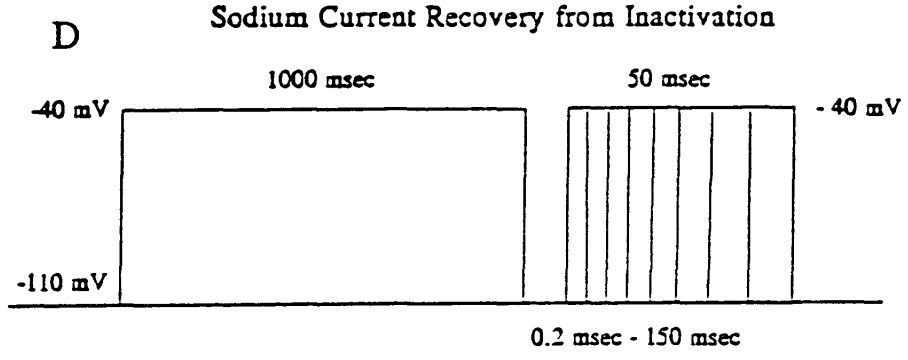
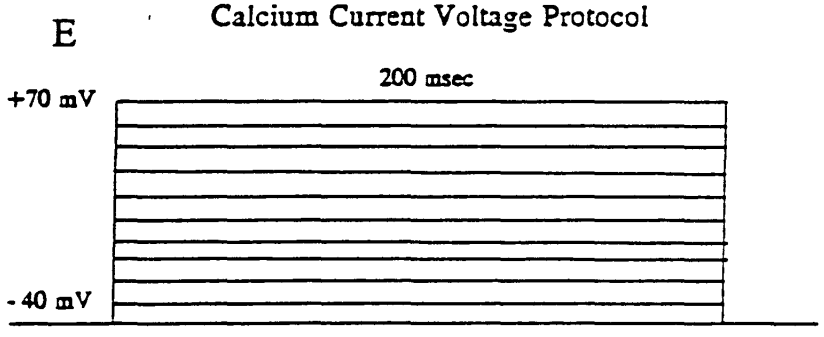


Figure 3
Experimental Voltage Protocols





Ca^{2+} Current. Ionic currents were measured with nystatin perforated-patch whole cell recording technique (Horn & Marty, 1988). Cells were held at -40 mV and clamped from -40 to +70 mV for 200 msec in 10 mV voltage steps. Peak $I_{Ca,L}$ was measured with respect to steady state current.

Action Potential and Cell Shortening. Intracellular recordings were obtained with the nystatin perforated patch technique. Cells were stimulated by an external source at variable frequencies at a stimulus amplitude sufficient to generate an action potential. Cell shortening was measure with a video edge detector by following the one edge of the contracting cell. Action potentials and cell shortening was recording on the computer as well as magnetic reel to reel tape.

G. Measurements and Data Analysis.

The membrane capacitance (C_m) was measured by delivering a ramp voltage clamp pulse (dV/dt=5V/sec) and dividing the half-amplitude of the current jump at the turning point of the ramp pulse by the ramp slope. Whenever possible the currents were normalized in reference to the C_m of each individual cell to yield current density (pA/pF). Data are presented as means $\pm$ standard error of the mean (SEM). Paired t-test was used for statistical analysis and differences with p values <0.05 were considered statistically significant.

H. Solutions, Drugs and Chemical Reagents.

All solutions were made on the same day as the experiment from double-deionized water. The solutions used for cell isolated are listed in Table 1. The internal pipette solutions used for standard ruptured patch and nystatin-perforated patch are listed in Table 3.

List of drugs and chemical reagents:

Collagenase (type II, 209 units/mg)

Albumin (Sigma Chemical Company)

Na_2-ATP (grade 1, Sigma Chemical Company)

Ethyleneglycol-bis-N,N,N',N'-Tetraacetic Acid (Sigma Chemical Company)

Nystatin (Sigma Chemical Company)

Tetrodotoxin (Calbiochem)

4-Aminopyridine (Sigma Chemical Company)

Tetraethylammonium Chloride (Sigma Chemical Company)

Ryanodine (Penick, Lyndhurst, New Jersey)

Verapamil (Sigma Chemical Company)

3,5,3'-Triiodo-L-thyronine (Sigma Chemical Company)

3,3',5'-Triiodo-L-thyronine (Sigma Chemical Company)

3,5,3',5'-Tetraiodothyronine (Sigma Chemical Company)

Thapsigargin (Sigma Chemical Company)

Table 1. Cell Isolation Solutions

	1 Normal Tyrode	2 Ca^{2+}-free Tyrode	3 Perfusion Enzyme	4 HEPES Tyrode
NaCl (mM)	137	137	137	137
KCl (mM)	5.4	5.4	5.4	5.4
$CaCl_2$ (mM)	1.8	--	0.036	1.8
$MgCl_2$ (mM)	1.0	1.0	1.0	1.0
$NaHCO_3$ (mM)	12	12	12	--
NaH_2PO_4 (mM)	0.6	0.6	0.6	--
Glucose (mM)	11	11	11	11
Albumin (%)	--	--	0.1	--
Collagenase (%)	--	--	0.06	--
HEPES (mM)	--	--	--	5
pH	7.4*	7.4*	7.4*	7.35**

* Biocarbonate-buffered Tyrode solutions were bubbled with 95% O_2-5% CO_2 to pH 7.4.

** Hepes-buffered Tyrode solution was titrated with NaOH to pH 7.35.

Table 2. Cell Perfusion Solutions

	1 HEPES Tyrode	2 Low Na^+ Tyrode	3 High Cs^+ Tyrode
NaCl (mM)	137	50	137
KCl (mM)	5.4	5.4	5.4
$CaCl_2$ (mM)	2.0	2.0	2.0
$MgCl_2$ (mM)	1.0	1.0	1.0
TEA-Cl (mM)	--	67	--
Verapamil (mM)	--	0.005	--
4-Aminopyridine (mM)	--	2.0	--
CsCl (mM)	--	20	20
Glucose (mM)	11	11	11
HEPES (mM)	5	5	5
pH	7.35*	7.35**	7.35*

* Hepes-buffered Tyrode solution was titrated with NaOH to pH 7.35.

** Hepes-buffered Low Na^+ Tyrode solution was titrated with CsOH to pH 7.35.

Table 3. Internal Pipette Solutions

	1 Rupture Patch (I_{Na})	2 Nystatin Perforated Patch (I_{Ca})	3 Nystatin Perforated-Patch (A.P.)
Glutamic Acid (mM)	100	--	--
Cesium Glutamate (mM)	--	100	--
Potassium Glutamate (mM)	--	--	100
$MgCl_2$ (mM)	1.0	1.0	1.0
KCl (mM)	--	--	40
Na_2ATP (mM)	4.0	4.0	4.0
EGTA (mM)	10	0.5	0.5
CsCl (mM)	40	40	--
CsOH (mM)	100	--	--
HEPES (mM)	10	10	10
pH	7.20*	7.20*	7.20**

* Hepes-buffered Low Na^+ pipette solution and Ca^{2+} pipette solution were titrated with CsOH to pH 7.20.

** Hepes-buffered pipette solution (A.P.) was titrated with KOH to pH 7.20.

CHAPTER IV

RESULTS

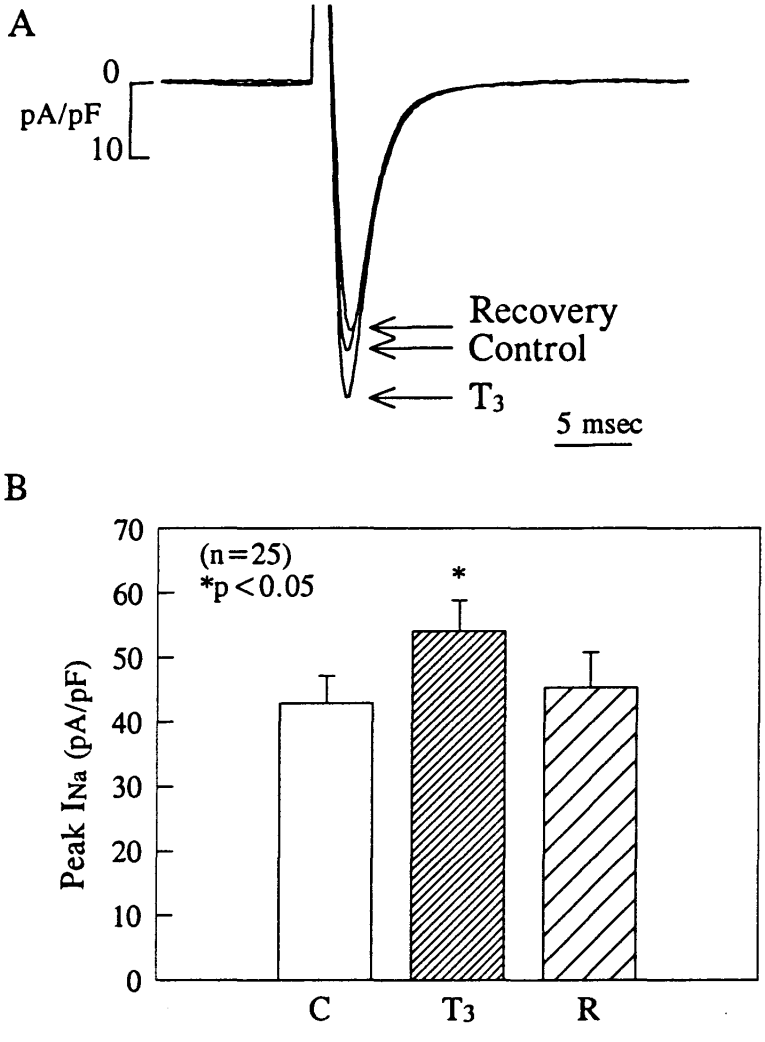
To be assured that the Na^+ current observed in isolated atrial myocytes were reasonable, the quality of the voltage clamp was evaluated. To monitor voltage clamp control two criteria were used. First, the voltage clamp step measured by the electrode was compared with the voltage clamp step generated by the computer to minimize the variation. Second, the current generated did not exhibit a "threshold phenomenon" near the voltage for peak current activation, but rather the current elicited a gradual increase as the clamp steps approached the peak current level. To aid in controlling the large Na^+ current, the $[Na]_o$ was reduced to 50 mM and all the Na^+ current experiments were conducted at room temperature.

A. Effect of Thyroid Hormone on Na^+ Current.

Macroscopic Na^+ current was recorded from isolated atrial cells using the ruptured patch technique. Figure 4A shows the peak inward Na^+ current elicited from an atrial muscle cell during a depolarizing clamp step from -80 mV to -40 mV under control conditions and when the cell was acutely (5 minutes) perfused with T_3 (10 nM). Under control conditions the peak inward current was -36.2 pA/pF while after exposure to T_3 the

Figure 4

Effect of T_3 on Sodium Current in Atrial Myocytes

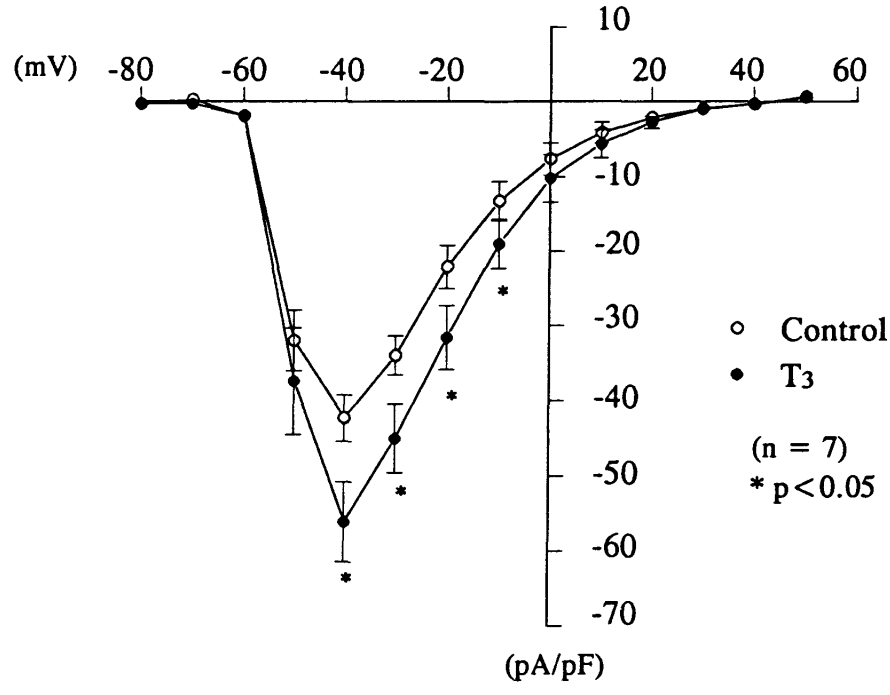


Effect of T_3 (10 nM) on atrial muscle cells. Panel A shows the peak inward sodium current elicited from a single atrial muscle cell in response to an acute exposure of T_3. Panel B shows the effect of an acute exposure on 25 cells and upon recovery (R) in 9 cells.

Figure 4 - continued

Effect of T_3 on Sodium Current in Atrial Myocytes

C



Panel C shows the effect of T_3 (10 nM) on the sodium current-voltage relationship.

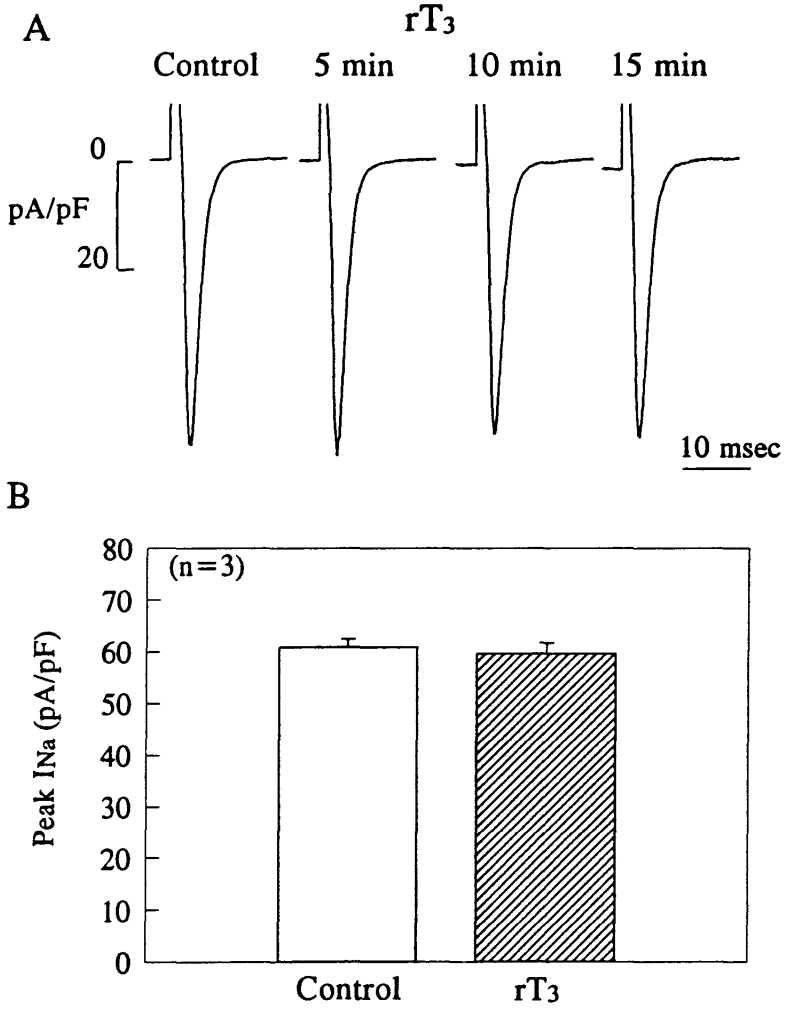
peak inward Na^+ current increased to -42.4 pA/pF. Upon washout of T_3, the current decreased to -33.4 pA/pF. In 25 cells tested (Figure 4B) the peak inward Na^+ current increased 26.1% from -42.9 $\pm$ 4.2 pA/pF under euthyroid conditions to -54.1 $\pm$ 4.7 pA/pF after an acute exposure to T_3. In 9 cells, after washout of T_3 for 5 minutes, the current returned to -45.4 $\pm$ 4.3 pA/pF indicating that T_3 was able to be virtually removed from the plasma membrane receptors or that by this time in the experiment (25 minutes) the cell had deteriorated such that the Na^+ current had rundown more even with T_3 still bound to its receptors.

When the current-voltage relationship (Figure 4C), generated by 10 mV incremental clamp steps from -80 mV to +50 mV (Figure 3A), was investigated in 7 cells, the onset of inward current (-60 mV), the voltage generating maximal inward current (-40 mV) and the reversal potential (+41.7 mV) all showed no variation between control conditions and when T_3 was applied. The Na^+ current showed an apparent reversal potential of +41.7 $\pm$ 1.5 mV, close to the theoretical reversal potential value of +46.7 mV calculated using the Nerst equation for a channel strictly passing Na^+ current, giving indication that the current elicited was the Na^+ current.

To investigate whether the increase in peak inward Na^+ current was specific to T_3 or showed similar responses to the other hormones produced by the thyroid gland, rT_3 and T_4 were investigated. Figure 5A shows the current elicited in a single clamp step from -80 mV to -40 mV in an atrial muscle cell in response to exposure to rT_3 (10 nM). In 3 cells tested (Figure 5B), the peak inward Na^+ current showed no statistically significant

Figure 5

Effect of rT_3 on Peak Inward Sodium Current in Atrial Myocytes

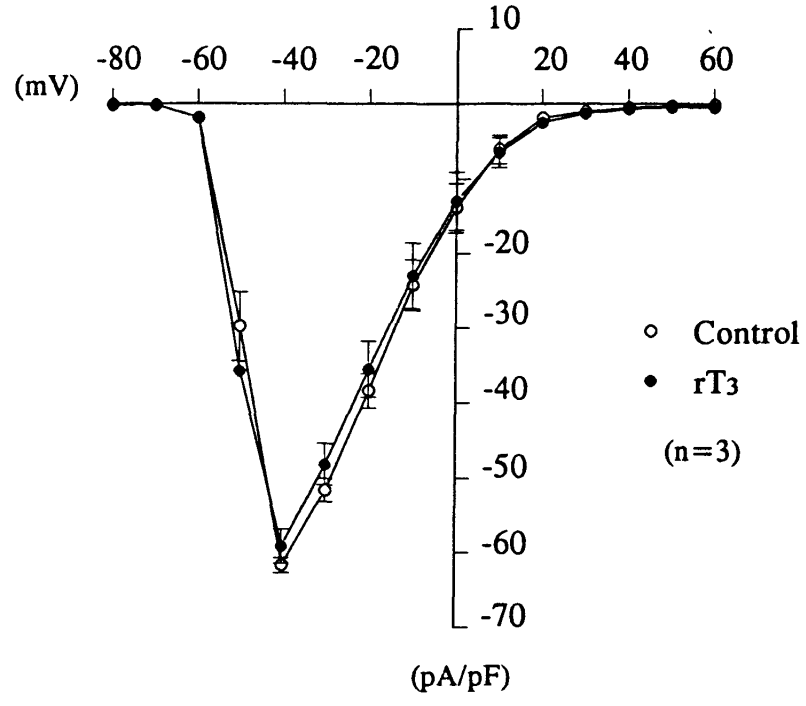


Effect of rT_3 (10 nM) on atrial muscle cells. Panel A shows the effect on the peak inward sodium current upon exposure to rT_3 in a single atrial muscle cell. Panel B summarizes the effect of rT_3 on the peak inward sodium current in 3 cells.

Figure 5 - continued

Effect of rT_3 on Sodium Current in Atrial Myocytes

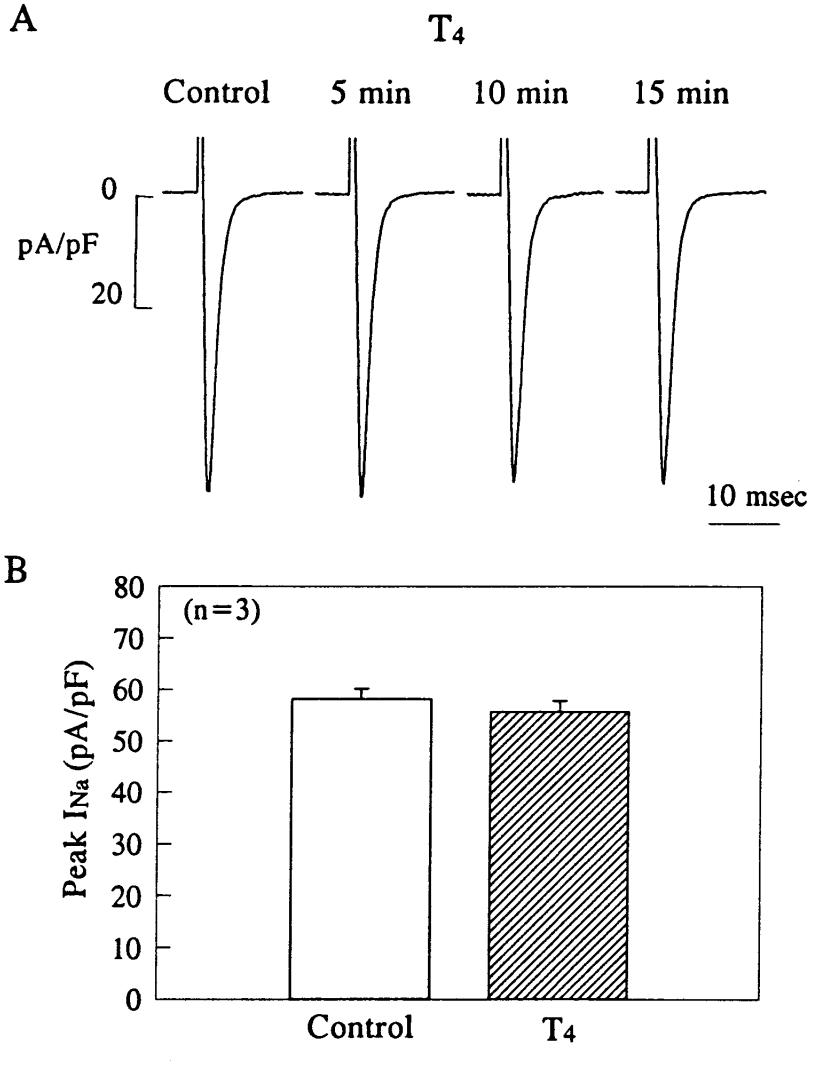
C



Panel C shows the effect of rT_3 (10 nM) on the sodium current-voltage relationship.

Figure 6

Effect of T_4 on Peak Inward Sodium Current in Atrial Myocytes

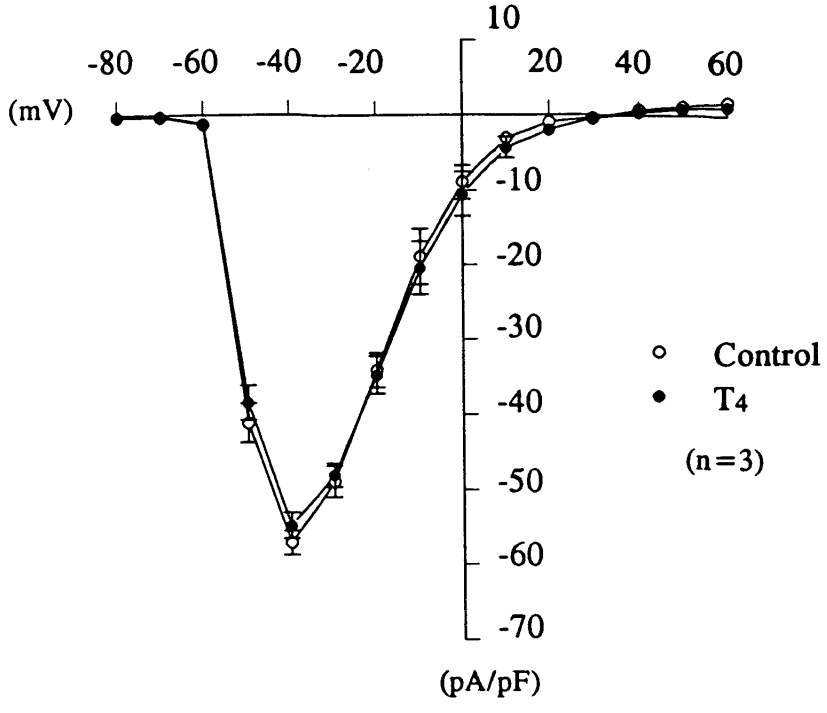


Effect of T_4 (10 nM) on atrial muscle cells. Panel A shows the effect on the peak inward sodium current upon exposure to T_4 in a single atrial muscle cell. Panel B summarizes the effect of T_4 on the peak inward sodium current in 3 cells.

Figure 6 - continued

Effect of T_4 on Sodium Current
in Atrial Myocytes

C



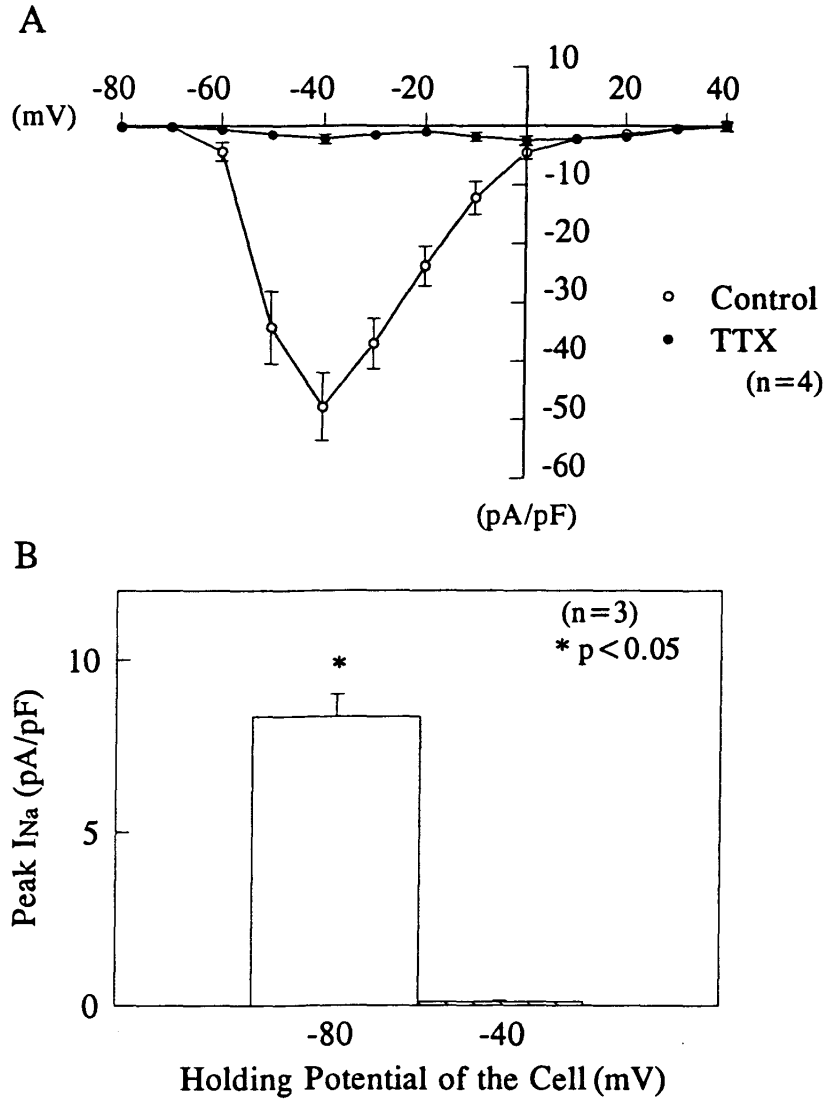
Panel C shows the effect of T_4 (10 nM) on the sodium current-voltage relationship.

variation between control (-60.9 ± 1.6 pA/pF) and rT_3 (-59.7 ± 2.2 pA/pF). The current-voltage relationship (Figure 5C) corroborated the peak current data, further demonstrating that rT_3 did not alter the threshold for channel activation, the voltage of peak current generation, or the reversal potential, indicating in our experiment design rT_3 failed to produce effects similar to T_3. Likewise, the effects of T_4 on atrial Na^+ current were investigated. As shown in figure 6A & 6B, the peak inward Na^+ current exhibited no significant change with T_4 (control: -58.2 ± 2.0 pA/pF vs. T_4: -55.7 ± 2.2 pA/pF). Similarly the current-voltage relationship (Figure 6C) did not vary with T_4 application, in its onset of current activation, its voltage of peak current or its reversal potential, giving indication that the increase in Na^+ current was specific to T_3.

To demonstrate that the current being elicited from the voltage clamp protocol was the "fast" Na^+ current, TTX (tetrodotoxin) along with variations in the holding potential of the cell were employed. Figure 7A demonstrates that 10 μM TTX was able to block 95.6% of the current as compared to control conditions (-2.2 ± 0.8 pA/pF vs. -47.9 ± 5.8 pA/pF). In figure 7B the holding potential of the atrial muscle cell was varied from -80 mV (HP-80) to -40 mV (a level believed to inactivate the sodium current). When the cell was clamped from these holding potentials to 0 mV the current generated at HP-80, -8.4 ± 0.6 pA/pF, was nearly completely eliminated at HP-40, -0.11 ± 0.05 pA/pF. These two results suggest that the current elicited by the voltage protocol is strictly the "fast" Na^+ current.

Figure 7

Effect of TTX on the Sodium Current
in Atrial Myocytes



Demonstration that the current elicited from the voltage clamp protocol (Figure 3A) is the "fast" sodium current. Panel A shows the effect of TTX (10 uM) on the current-voltage relationship. Panel B shows the effect of varying the holding potential (HP) of the cell on a single clamp step from HP to 0 mV.

B. Effect of T_3 on Na^+ Channel Kinetics.

To uncover the possible mechanism responsible for the increase in Na^+ current, channel kinetics were investigated. The steady state properties of Na^+ current inactivation were studied using a standard two-pulse protocol (Figure 3B). First a step of variable amplitude and a duration long enough to activate the Na^+ current were applied. Atrial cells were clamped from prepulses to selected conditioning potentials from a holding potential of -120 mV positive to +20 mV. After repolarization to the holding potential for 2 msec, a test pulse to -40 mV was applied. The current elicited by the test pulse was normalized to that obtained in the absence of a preceding conditioning pulse and compared in the presence and absence of T_3.

The steady state properties of Na^+ current activation were studied by measuring tail currents (Figure 3C). The atrial cell membrane was depolarized from -80 mV to a conditioning potential for a time long enough to allow the activation process to obtain its steady state value. The duration of the conditioning clamp was short enough so that inactivation did not have time to develop. At the end of the conditioning pulse the membrane was clamped to a fixed test potential and the amplitude of the tail current at the beginning of this step was measured. Atrial cells were held at -80 mV and clamped for approximately 2 msec, or a clamp duration not leading to channel inactivation, in 10 mV voltage steps to +50 mV. After the conditioning prepulse to various voltages, the cell was clamped back to -60 mV and the tail current was measured. Steady state activation was determined by comparing the tail current elicited at each varying prepulse with the

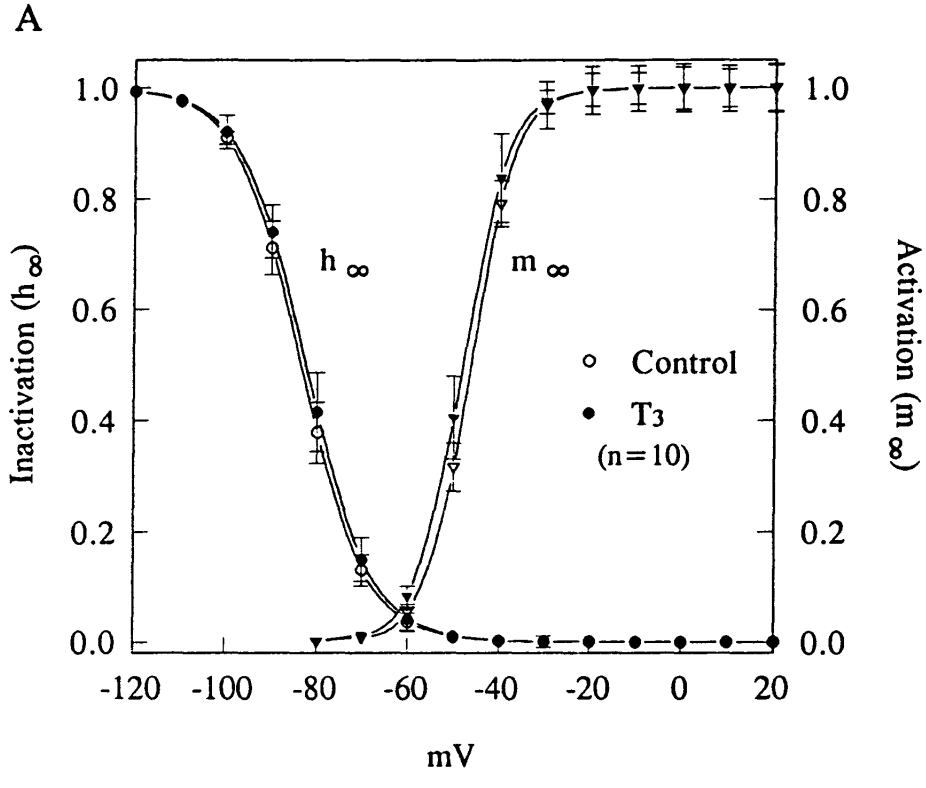
maximum current elicited, yielding a normalized steady state activation curve.

Figure 8A shows inactivation/activation curves in the absence and presence of T_3 (10 nM). In 10 cells tested there was no statistically significant increase in either parameter with T_3. Data were fitted by curves calculated using the Boltzmann function : $I_t/I_{max} = \{1 + \exp[(V_c-V_{0.5})/k]\}^{-1}$, where V_c is the voltage of the conditioning pulse, $V_{0.5}$ is half maximal voltage, and k is the slope factor. At -110 mV, h_∞ was 0.994 $\pm$ 0.006; at -40 it was 0.002 $\pm$ 0.005. The voltage of half-activation was -83.5 mV in control conditions versus -82.5 mV in the presence of T_3. The slope factor, k, was 7.1 under control and T_3 conditions. Under control and T_3 conditions, steady state activation had an initial onset of -60 mV and reached a peak channel activation at -20 mV. The "window" current, which is considered to be the steady state component of the "fast" Na^+ current resulting from the crossover of the activation and inactivation curves which govern the opening of the Na^+ channel, did not change with T_3 exposure.

The time course of Na^+ current recovery from inactivation was defined using the two-pulse protocol shown in figure 3D. Cells were held at -110 mV and clamped for a 1 second conditioning pulse to -40 mV. Following the conditioning pulse was a variable time interval (from 0.2 msec to 150 msec) at the holding potential after which the cell was clamped to -40 mV for 50 msec. The test current was compared with the conditioning current (I_{test}/I_{cond}) at each recovery time. Na^+ channel recovery from inactivation (Figure 8B) was investigated in 6 cells. There was no statistical variation in channel recovery rate in the absence or presence of T_3. In both cases the channel reached 90% recovery in

Figure 8

Effect of T_3 on Sodium Channel Kinetics
in Atrial Myocytes

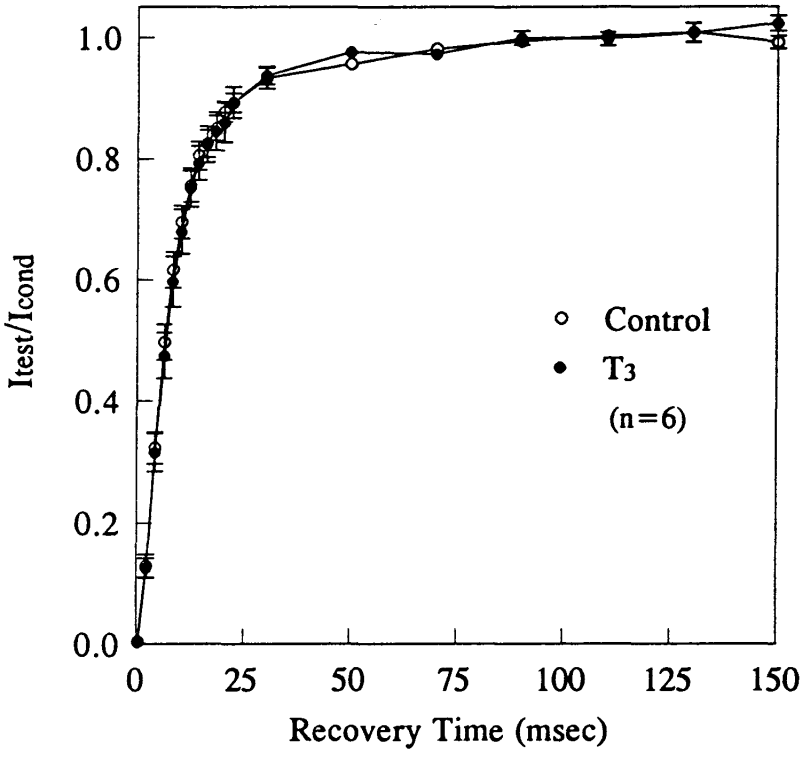


Voltage-dependence of activation and inactivation for sodium current. Open symbols represent steady-state activation and inactivation curves for control. Closed symbols represent the steady-state activation and inactivation for T_3 (10 nM). Continuous curves were obtained by fitting the data with a Boltzmann equation.

Figure 8 - continued

Sodium Channel Recovery from Inactivation
in Atrial Myocytes

B



Effect of T_3 (10 nM) on the sodium channel recovery time from inactivation. Test current peak (I_{test}) normalized versus conditioning current peak (I_{cond}) under control conditions and after an acute exposure to T_3.

approximately 25 msec.

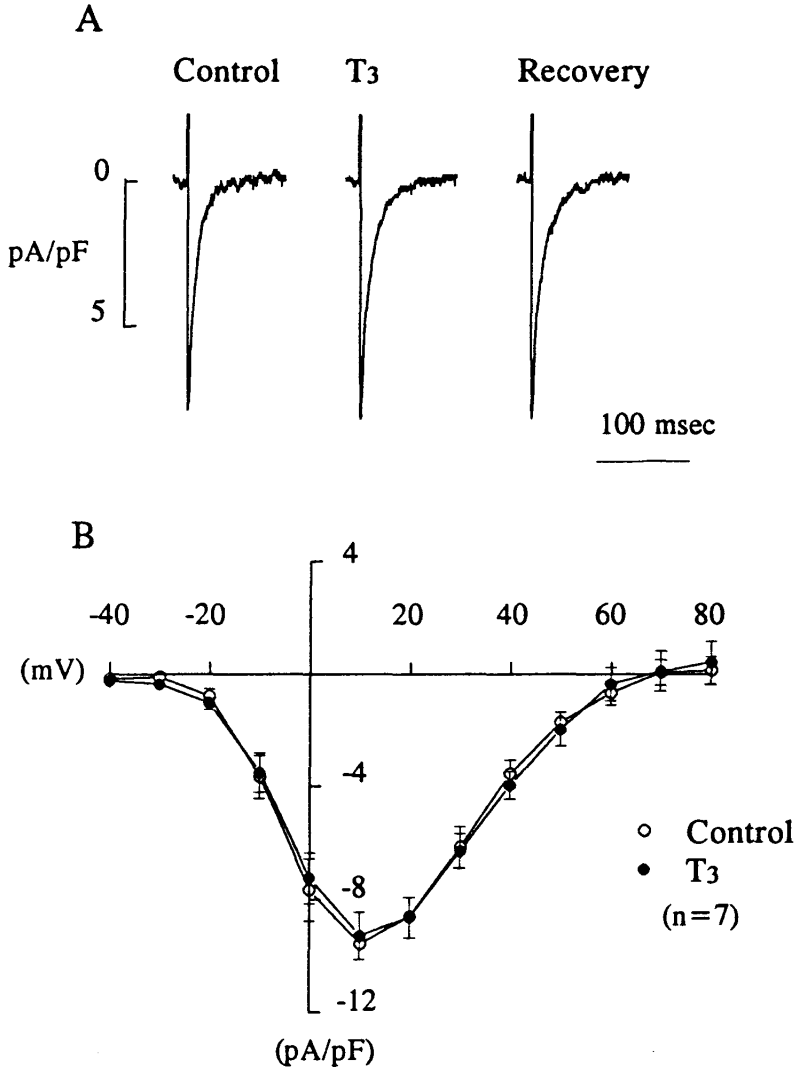
C. Effect of T_3 on Ca^{2+} current.

Other researchers have documented a chronic increase in the L-type Ca^{2+} channel current in the presence of thyroid hormone (Binah *et al.*, 1987) as well as an acute increase in calcium uptake (Gøtzsche, 1994; Segal, 1989). In this experimental design, in the time demonstrated to increase the Na^+ current (0-15 minutes) there was no increase in the peak Ca^{2+} current (Figure 9A) or in the current-voltage relationship (Figure 9B). In 7 atrial muscle cells tested the onset of the current, the voltage of peak current, and the reversal potential of the current all remained unchanged when comparing control and T_3 (10 nM).

The T-type Ca^{2+} current may be influencing the increase seen with T_3 when measuring the Na^+ current. To investigate this possibility, $NiCl_2$ (50 μM), a specific blocker of the T-type Ca^{2+} current (Wu & Lipsius, 1990) was perfused. Initially the cell was exposed to T_3 and the voltage protocol used to measure the Na^+ current (Figure 3A) was employed. After an increase in Na^+ current was seen with T_3, $NiCl_2$ + T_3 was perfused and the current was recorded. In 5 atrial cell after 5 minutes of perfusion with $NiCl_2$ there was no statistically significant decrease in the peak inward Na^+ current (Figure 9C) (Control: -45.9 ± 2.3 pA/pF, T_3: -55.2 ± 3.8 pA/pF, T_3 + $NiCl_2$: -52.9 ± 3.0 pA/pF). These results indicate that the T-type Ca^{2+} current was not influencing the increase seen in the Na^+ current with exposure to T_3.

Figure 9

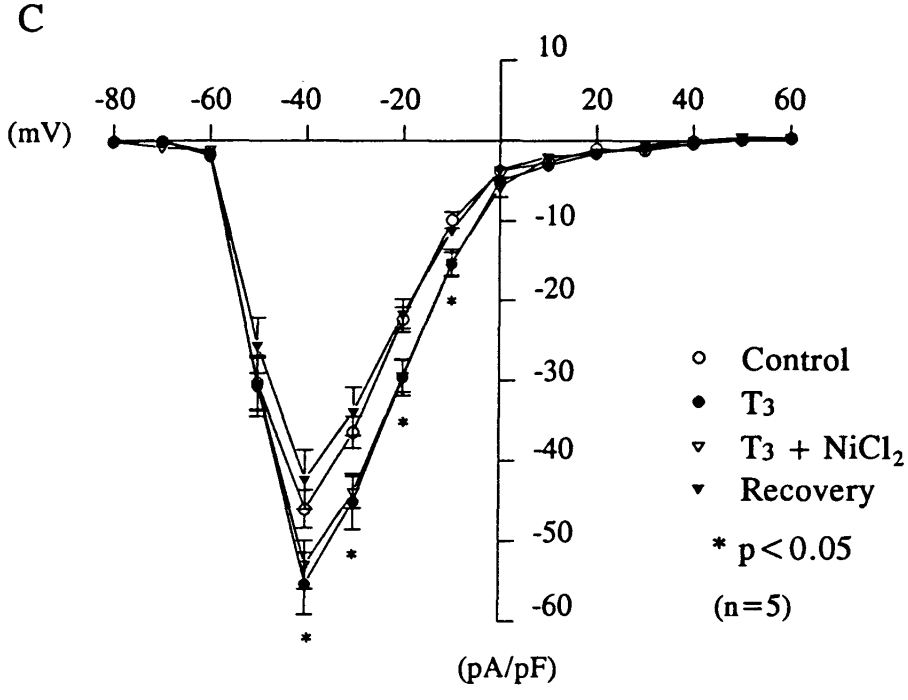
Effect of T_3 on L-type Calcium Current in Atrial Myocytes



Effect of T_3 (10 nM) on the L-type calcium current in atrial muscle cells. Panel A shows the peak inward calcium current in control, T_3, and recovery. Panel B shows the current-voltage relationship for the L-type calcium current under control conditions and after acute T_3 exposure.

Figure 9 - continued

Influence of T-type Calcium Current on
Sodium Current Increase with T_3
in Atrial Myocytes



Influence of the T-type calcium current on the sodium current increase with T_3 (10 nM) in atrial muscle cells. Sodium current-voltage relationship under control conditions, after an acute exposure to T_3, after subsequent exposure to T_3 + $NiCl_2$ (50 uM) and after recovery.

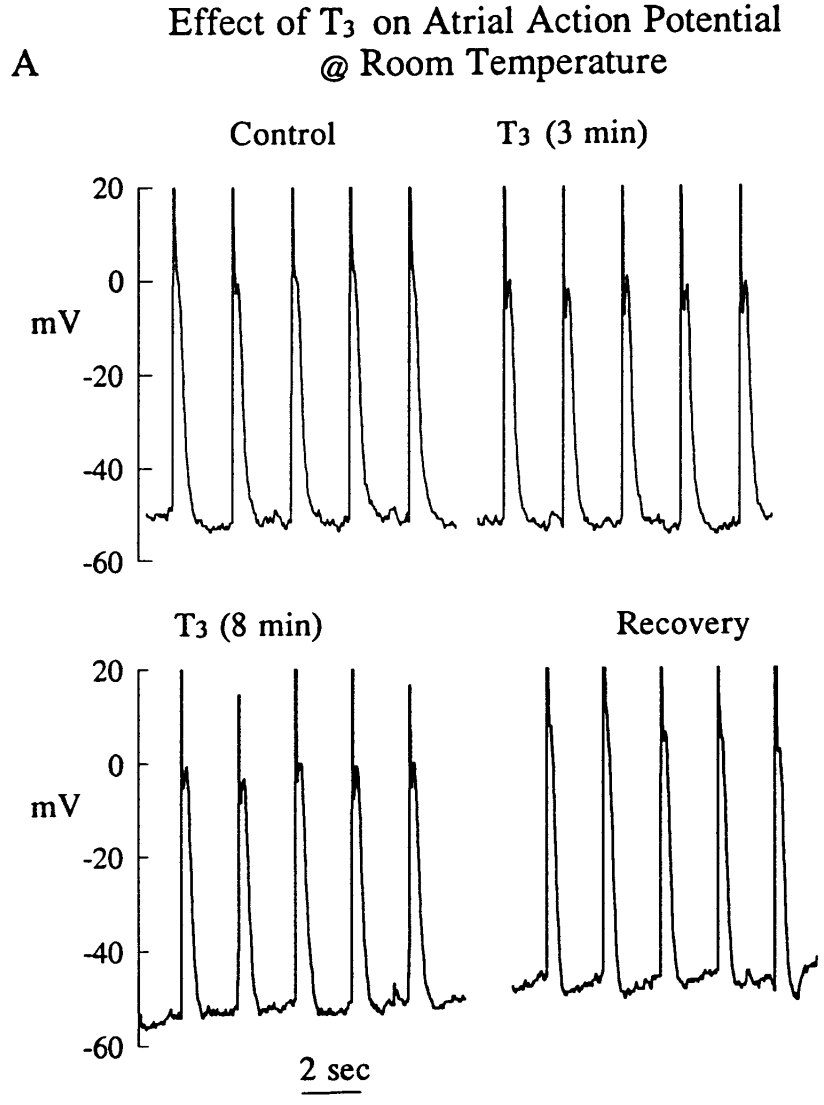
D. Effect of T_3 on Action Potential Properties.

Correlating the changes seen in the Na^+ current data, stimulated (0.5 Hz) atrial muscle cell action potentials were investigated. In figure 10A & B the effect of T_3 on action potentials in a single atrial muscle cell at room temperature were investigated. Exposure to T_3 (10 nM) lead to a gradual "notching" of the plateau phase of the action potential, in 3 of 5 cells tested. Upon withdrawal of T_3, in all 3 cells, this "notching" disappeared. The resting membrane potential and peak action potential overshoot were unaffected by T_3 exposure. Action potential duration (90% repolarization) decreased with T_3 exposure from 525.4 $\pm$ 7.0 msec to 479.2 $\pm$ 9.0 msec. Interestingly the duration of 50% repolarization slightly increased with T_3 from 233.6 $\pm$ 8.0 msec to 248.4 $\pm$ 10.4 msec due probably to the "notching" seen during the plateau phase of the action potential.

Figure 11 shows the effects of T_3 on stimulated (0.5 Hz) atrial muscle cells at 35°C. Exposure to T_3 (10 nM) lead to spontaneous depolarizations which increased in frequency with the length of the T_3 perfusion. In 7 out 10 cells tested, perfusion with T_3 lead to the generation of spontaneous beats. Of the cells that exhibited spontaneous beats, the average time before spontaneous beats developed was 4.9 $\pm$ 0.9 minutes with a range from 1 to 8 minutes. After 5 minutes of perfusion at 35°C, the cells exhibited on average 10.4 $\pm$ 1.4 extra beats in a 40 second period. Upon withdrawal of T_3 in 5 out of 7 of these cells the spontaneous beats disappeared.

In two series of experiments, the effect of SR load was investigated to determine its effect on the T_3 induced delayed after depolarizations. Ryanodine, an alkaloid that

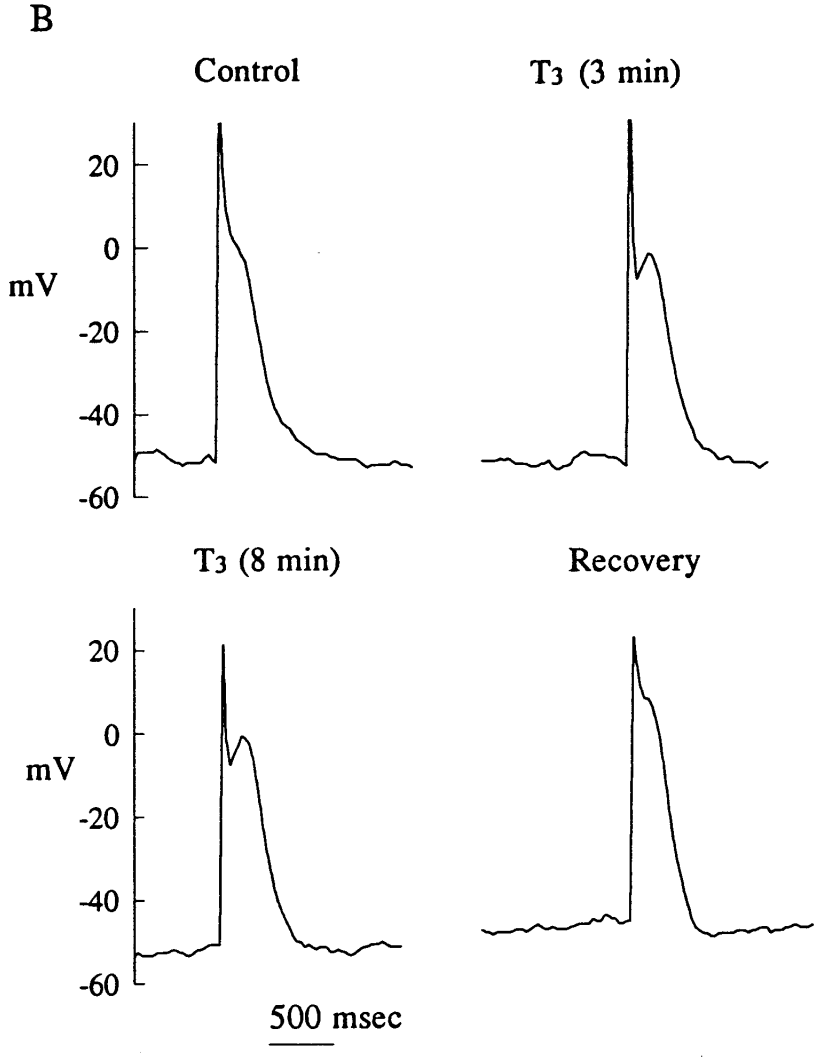
Figure 10



Action potential recordings from an atrial muscle cell stimulated at 0.5 Hz at room temperature. Recordings during control, T_3 (10 nM), and recovery.

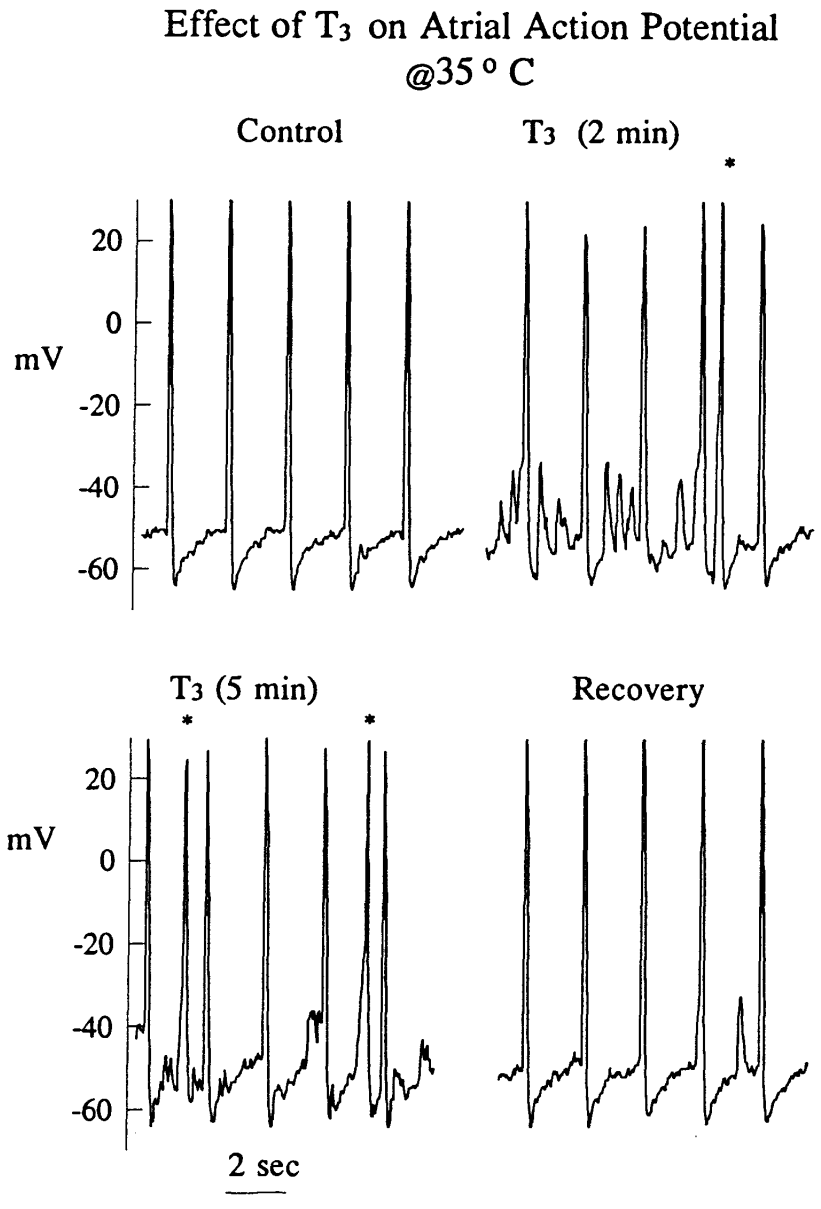
Figure 10 - continued

Effect of T_3 on Atrial Action Potential
@ Room Temperature



Panel B shows individual action potentials in atrial muscle cells stimulated at 0.5 Hz at room temperature. Recordings during control, T_3 (10 nM), and recovery.

Figure 11



Action potential recordings from an atrial muscle cell stimulated at 0.5 Hz at 35 ° C. Recordings during control, T3 (10 nM), and recovery. Spontaneous beats are indicated by (*).

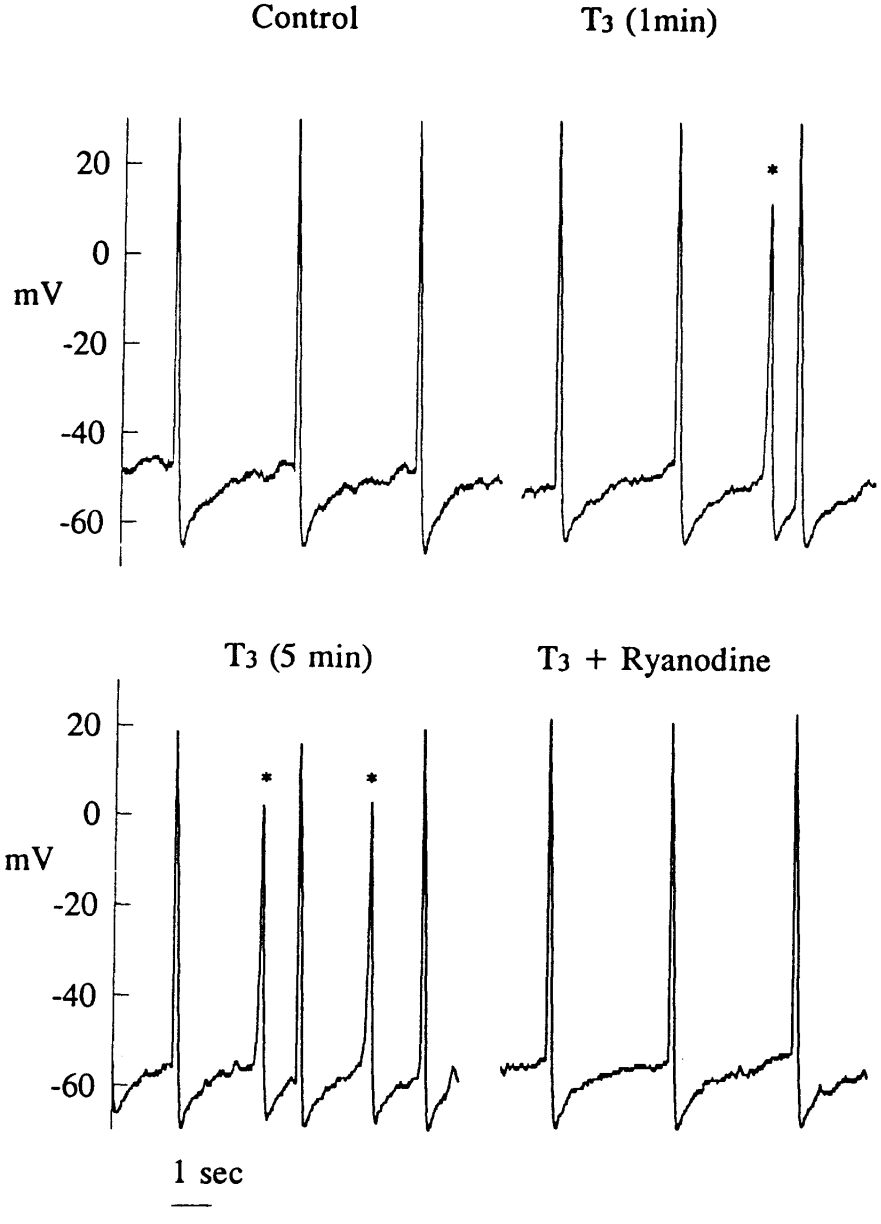
opens SR Ca^{2+} release channels (Janczeski & Lakatta, 1993) and prevents accumulation of SR Ca^{2+} and thapsigargin, an inhibitor of SR Ca^{2+} uptake (Rousseau *et al.*, 1987) were tested. Figure 12 shows that the effects of ryanodine on stimulated atrial muscle cells in the presence of T_3. In 4 atrial cells demonstrating spontaneous beats with T_3, ryanodine (1 μM) in the presence of T_3 lead to the elimination of the extra beats. Similarly, thapsigargin (5 μM) also eliminated the extra beats in the presence of T_3 alone (data not shown). During control conditions, resting membrane potential was -56.4 ± 0.5 mV as compared to -55.0 ± 0.7 mV when perfused with T_3. Likewise the action potential overshoot showed no statistically significant variation with T_3 (control: 23.6 ± 0.2 mV vs. T_3: 25.5 ± 1.7 mV). These results indicate that by inhibiting the accumulation of SR Ca^{2+}, these agents selectively abolished the proarrhythmic effects of T_3.

E. Effect of T_3 on Cell Shortening

Finally the contractile behavior of single atrial cells was investigated (Figure 13). Cell contractile strength was quantified by measuring unloaded cell shortening in response to a stimulus sufficient to generate an action potential. A video edge detector was used to track the edge of one edge of the cell to quantify the degree of cell shortening. Figure 13 depicts simultaneous action potential recordings (A) and cell shortening (B) under control, T_3, and recovery conditions. In 5 atrial muscle cells the cell shortening increased 34.8 ± 1.6 % when T_3 was perfused as compared with control. Upon washout of T_3 for 5 minutes, contractility returned to cell shortening 8.4 ± 1.1% above control

Figure 12

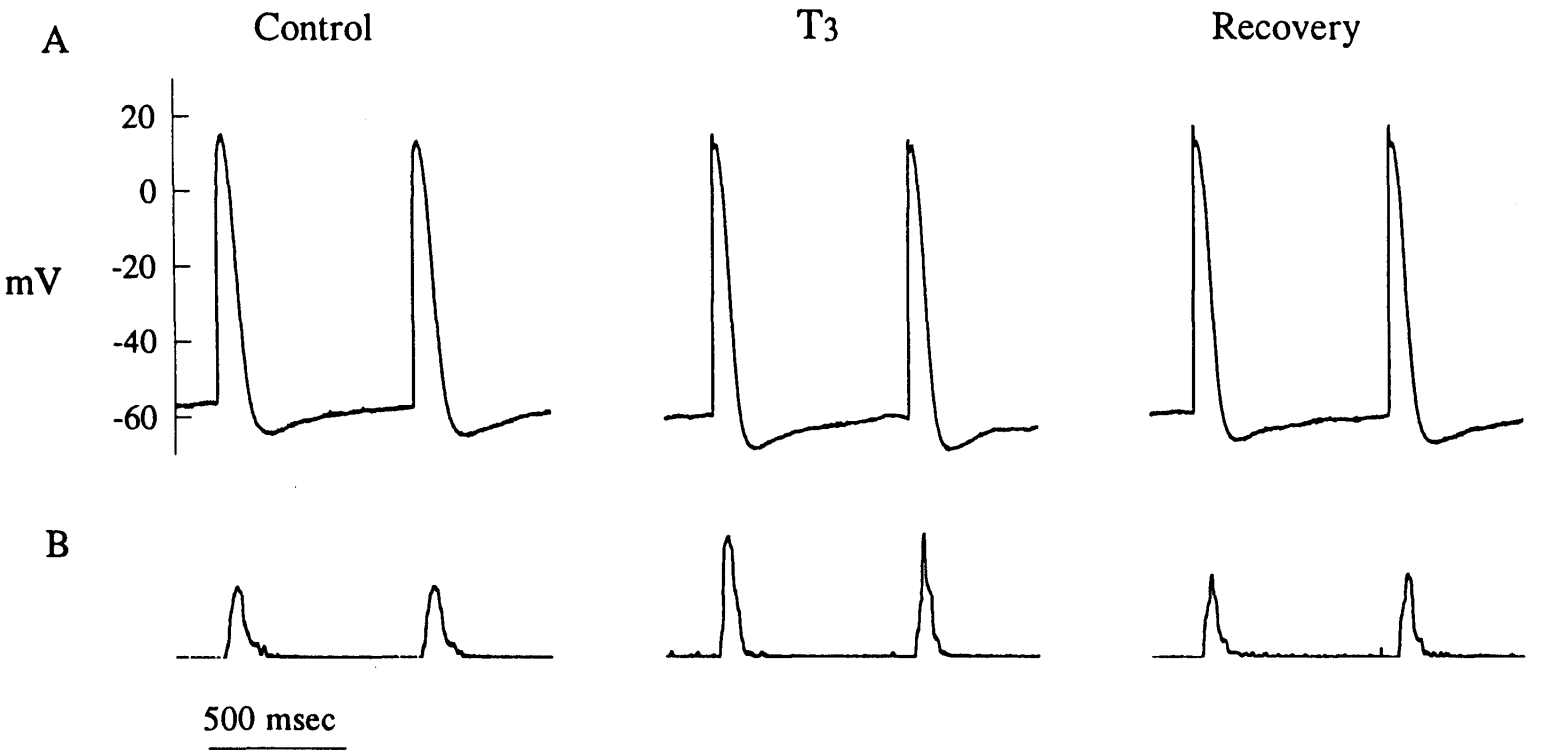
Effect of Ryanodine on Action Potentials in Atrial Myocytes



Influence of SR load on T3 (10 nM) induced arrhythmias. Action potentials were recorded from atrial muscle cells at 35° C, under control, T3, and after T3 + Ryanodine (1 uM). Spontaneous beats are indicated by the (*).

Figure 13

Effect of T_3 on Action Potentials and Cell Shortening in Atrial Myocytes



Panel A shows action potential recordings at 35° C in control, T_3, and recovery conditions in an atrial muscle cell. Panel B shows simultaneous cell shortening measurements under control, T_3, and recovery.

levels indicating that T_3 still had lingering effects due probably to slow dissociation of T_3 from the plasma membrane receptors.

CHAPTER V

DISCUSSION

The main purpose of the present study was to determine the acute (0-15 minute) effects of thyroid hormone (T_3) on atrial muscle cells. Cardiac muscle cells were isolated from the cat atrium and the properties of the Na^+ current, the Ca^{2+} current, action potentials and cell shortening generated by stimuli were investigated in the presence of thyroid hormone (T_3). Investigation into an individual ionic current was accomplished by isolation of a particular current through a specific voltage clamp protocol and by blocking currents other than the one of interest by use of specific channel blocking agents (i.e. 4-aminopyridine, verapamil, Ni^{2+}). Na^+ current was measured by the ruptured patch whole cell recording technique to decrease access resistance to the cell while Ca^{2+} current and action potential recordings were measured with the nystatin-perforated patch technique to minimize cell dialysis of intracellular constituents with the internal pipette solution and to decrease current rundown.

In these experiments, T_3 at a dose (10 nM) comparable with research conducted by other investigators was perfused around the isolated cells to produce the acute hyperthyroid state. In humans the normal circulating serum thyroid hormone (T_3) levels

are in the nanomolar range (2.33 ± 0.54 nM), and patients experience the clinical signs of hyperthyroidism at slightly higher levels (5.08 ± 1.60 nM)(Galløe *et al.*, 1993). With the biologically active free T_3 levels only 0.2-0.3% of the serum levels (Berne & Levy, 1993), the use of 10 nM in our experiments might at first seem like a high hormone concentration. A number of things must be kept in mind.

First, we are investigating the effects of thyroid hormone *in vitro* and not *in vivo*. There could be other mitigating factors not present in our system which are present in the intact animal (human) where by levels of thyroid hormone near the cell surface might be much greater than blood levels detected by common clinical procedures. In chronic thyroid hormone experiments, researcher have shown a preferential uptake of thyroid hormone into the cell, indicating that the cell can create a concentration gradient with higher concentrations of thyroid hormone inside the cell than outside in the bathing medium (De Nayer, 1989; Pontecorvi & Robbins, 1986; Krenning *et al.*, 1981; Oppenheimer & Schwartz, 1985). Therefore, it is not unreasonable to believe that T_3 bound to the plasma membrane receptors may be at a higher concentration than the free circulating levels in the blood. To date there is no known study looking at the concentration of thyroid hormone bound to the plasma membrane surface in relation to the concentration measured in the blood.

Second, thyroid hormone has been shown to bind to various plastics (Sypniewski, 1993). Since the perfusion apparatus and tubing leading to the cell bath are made from plastic solution reservoirs and polyethylene tubing (PE tubing), it may be possible that

the actual concentration of thyroid hormone at the cell surface might be lower than the concentration of thyroid hormone in the stock perfusion solution.

Third, the cell isolation procedure may have disrupted at portion of the thyroid hormone plasma membrane receptors, requiring a higher concentration of T_3 to cause anoticeable effect on the Na^+ current than would have been needed with the full compliment of plasma membrane receptors. Segal and Ingbar (1986) have shown that trypsinization of the membrane can remove the functionality of the plasma membrane receptors. They demonstrated that trypsinization caused attenuation of binding and action of T_3 on the plasma membrane in rat myocytes. So it could be possible that the cell isolation procedure caused a disruption in the normal binding of T_3 with its plasma membrane receptors, requiring an increased concentration of T_3 to lead to a functional change upon binding to the plasma membrane receptors. Also, since the extracellular Na^+ concentration was reduced by $^1/_3$, to decrease the magnitude of the Na^+ current so the electrode could follow the voltage clamp step, the concentration of T_3 required to show a significant effect on the Na^+ current might need to be higher than during *in vivo* condition when the $[Na]_o$ is the typical 137 mM.

Characteristically, the plasma membrane-mediated effects of thyroid hormone are prompt in onset (seconds to minutes), thereby being independent of influencing new protein synthesis/degradation rates, and are associated with changes in the transmembrane transport of ions and substrates (Segal, 1989). In chronic hyperthyroidism, thyroid hormone binds to nuclear thyroid hormone receptors (*c-erb* A) altering gene

transcription, leading to changes in protein synthesis/degradation rates. Shanker *et al.* (1987), investigating the response of individual mRNAs to T_3, showed that in all but one translational product responsive to T_3 (n=13), the time require for induction was on the order of at least a couple of hours. In the translational product demonstrating the most rapid response to T_3, "spot 72b", within one hour of T_3 exposure, levels rose to 2.6 fold those of control. Oppenheimer demonstrated that this lag time between thyroid hormone application and response in mRNA levels was as short as 20 minutes for the appearance of the cytoplasmic mRNA for the protein termed "spot 14" (Oppenheimer, 1985). Still, one must remember that formation of mRNA is just one of the first few steps of protein synthesis. Before this up regulated mRNA level leads to an up regulated protein level affecting cell functional properties, protein translation, modification, translocation and possible membrane insertion must take place, all requiring additional time.

Further support for the action of T_3 not mediating its effects through a nuclear mechanism in our experimental design, comes from research conducted by Banerjee *et al.* (1988), who showed that nuclear receptors for thyroid hormone are lower in atrial tissue versus ventricular tissue. They showed that the binding capacity for T_3 nuclear receptors in the atrium was considerably lower than found in the ventricle. Maximum binding capacity was 170 ± 20 fmol/mg DNA for ventricular nuclei vs. 55 ± 10 fmol/mg DNA for atrial muscle cell nuclei (Banerjee *et al.*, 1988). In our experiments, cyclohexamide was not used to block protein synthesis, but due to the short time interval (under 15 minutes and in most cases under 10 minutes) between initial exposure to T_3 and

measurable effect on Na^+ current as well as the lower nuclear T_3 receptor number in atrial versus ventricular tissue, we are confident that the increase in the Na^+ current is not working through nuclear receptors up regulating protein synthesis rates but rather through a plasma membrane mediated mechanism.

In the present experiments thyroid hormone (T_3) was shown to acutely increase the Na^+ current in atrial myocytes. Using the ruptured patch technique which dialyses the cell with the internal pipette solution, Na^+ current increased (26.1%) within 10-15 minutes. In 11 control cells, the Na^+ current ran down less than 10% (-44.3 $\pm$ 4.5 pA/pF to -39.8 $\pm$ 4.1 pA/pF) over the time demonstrated to increase Na^+ current with T_3 in other cells. Therefore, in actuality the Na^+ current increase with T_3 is really an underestimate of the true magnitude of the effect of T_3 on I_{Na}. Other groups have shown a similar increase in macroscopic Na^+ current in neonatal myocytes (Craelius *et al.*, 1989) and an increase in single Na^+ channel bursting behavior in ventricular myocytes (Dudley and Baumgarten, 1993) with T_3 exposure. Upon washout of T_3, in 9 cells tested, Na^+ current returned to levels slightly above initial control levels, indicating either that T_3 was able to be removed from the plasma membrane receptors of the cells or that by this time (around 25 minutes) into the experiment the cells were deteriorating at a rate sufficient to decrease the magnitude of the Na^+ current.

The acute increase in Na^+ current was shown to be specific to T_3 as T_4 and rT_3, other hormones produced by the thyroid gland, exhibited no effect on the Na^+ current. In experiments similar to these, Sakaguchi *et al.*(1995) demonstrated that T_3 acutely

increased the inward rectifier potassium current (I_{K1}) but rT_3 did not potentiate the I_{K1} current, even partially inhibiting the effect of T_3 on increasing I_{K1}. In contrast with our results, other researchers have demonstrated an effect of T_4 on isolated cardiac cells on the expression of SR mRNA protein (Arai *et al.*, 1991), Na^+-Ca^{2+} exchanger expression (Boerth & Artman, 1996) and cardiac conduction (Shahawy *et al.*, 1975) though primarily the effect was seen in chronic T_4 exposure where peripheral conversion, by 5'-monodeiodinase, could still occur from T_4 to T_3.

To investigate the mechanism by which T_3 is generating an increase in the Na^+ current, Na^+ channel kinetics were investigated. Both the steady state activation and steady state inactivation curves did not change with T_3 indicating that there was no alteration in the gating mechanism of the channel with T_3. In an individual isolated current, the decay of the peak Na^+ current showed similar kinetics as control. In contrast to these findings, Craelius *et al.* (1989), found the decay from peak Na^+ current slowed with an acute T_3 exposure. In our findings the recovery of the channel from inactivation showed no difference with T_3. Because the ruptured patch technique of cellular recording was used, cellular constituents in the cytoplasm were dialyzed away removing their potential influence on the gating mechanism of the channel. But since the increase in Na^+ current was also demonstrated with the rupture patch technique, the lack of cellular constituents did not influence the effect of T_3 on increasing the Na^+ current indicating that a cytosolic second messenger is probably not involved in the upregulation of the Na^+ current with T_3. The lack of alterations in the sodium channel kinetics with T_3 indicate

that T_3 is not altering the voltage sensor of the channel or the ability of the channel to recover from the inactivated state.

For the macroscopic Na^+ current to increase, the number of functional Na^+ channels (N) could increase, the open probability of an individual channel (P_o) could increase, or the current (*I*) passing through an individual channel could increase. The proposed mechanism by which T_3 is increasing the macroscopic Na^+ current is by increasing the open probability of the channel. Previously, in single channel recordings, Dudley and Baumgarten (1993) showed in ventricular tissue that single channel current did not increase with T_3 but rather there was an increase in the bursting behavior of the channel. This does not discount the chance that T_3 may also be increasing the number of functional Na^+ channels, but due to the time frame (minutes) associated between application of T_3 and the measured cellular response, it seems unlikely that new channels are inserted into the membrane at such a fast rate.

It could be possible that T_3 is acting to "turn on" Na^+ channels already in the plasma membrane and thereby increasing the macroscopic current. Sen & Cui (1992) showed in ventricular myocytes that T_3 ($1x10^{-8}M$) acutely increased the TTX-sensitive I_{Na}. When GTP was loaded into the cells, the onset of increase in I_{Na} with T_3 occurred sooner and lead to a greater increase in the peak Na^+ current level. This effect was abolished when the cells were pretreated with pertussis toxin, giving indication that PTX sensitive G proteins may play an important role in the molecular mechanism underlying the regulation of Na^+ channels by T_3. Therefore T_3 may be acting through membrane-

delimited PTX sensitive G proteins (Wickman & Clapham, 1995) to activate Na^+ channels, leading to an increased macroscopic Na^+ current.

Segal and Gøtzche showed that T_3 acutely increased calcium uptake into the cell. Segal (1989) demonstrated that thyroid hormone acutely increased calcium ($^{45}Ca^{2+}$) uptake in thymocytes that was blocked with inorganic calcium channel blockers, while Gøtzche (1994) showed in a whole animal experiment that T_3 decreased calcium perfusate concentration (i.e. increased cellular calcium uptake) which was blocked with the calcium channel blocker, nifedipine. In chronic hyperthyroidism Binah *et al.* (1987) showed that peak Ca^{2+} current increased in ventricular myocytes. In contrast with these results, in atrial myocytes T_3 failed to elicit a different response in the Ca^{2+} current than under control conditions. In the time demonstrated to increase the Na^+ current (0-15 minutes) there was no increase in the Ca^{2+} current peak amplitude, nor a shift in the current-voltage relationship of the channel in response to T_3, indicating that the cellular effects of T_3 in these experiments were not influenced by changes in the L-type Ca^{2+} current.

The T-type Ca^{2+} current could also be influence by T_3. To investigate this possibility, recordings were performed on cells in the presence and absence of Ni^{2+} (50 μM), a specific blocker of the T-type Ca^{2+} current (Wu & Lipsius, 1990). The increase in Na^+ current demonstrated with T_3 was not decreased by perfusion of the cell with T_3 and Ni^{2+} giving indication that the T-type Ca^{2+} current was not involved in the acute cellular response to T_3. Even if the T-type Ca^{2+} current influenced the current increase with T_3, because the T-type Ca^{2+} current is so small in atrial myocytes, -1.0 ± 0.06 pA/pF

(Wu & Lipsius, 1990), the T-type Ca^{2+} current would have to increase at least 5-6 fold to account for the approximately 10 pA/pF increase (-42.9 to -54.1 pA/pF) seen in the current with T_3.

Correlating the cellular changes in the macroscopic Na^+ current with the functional properties of cardiac muscle cells, the effects of T_3 on atrial muscle cell action potential and contractile strength were investigated. At room temperature, acute T_3 exposure caused a decrease in the action potential duration. The plateau phase of the action potential exhibited a "notching" effect indicating a secondary increase in net inward current generated. Typically during the plateau phase of the action potential current influx is equal to current outflux, hence generating the plateau in the voltage recording. Sharp *et al.* (1985) showed a similar phenomenon of "double spikes" in the action potential recordings in the presence of chronic hyperthyroidism which was eliminated by the calcium channel blocker D-600, suggesting that the current responsible for this second depolarization was mediated by a voltage dependent Ca^{2+} conductance. The current responsible for "notching" seen in our experiments was not fully investigated but thoughts are that it may be an increased Ca^{2+} influx through the Na^+-Ca^{2+} exchanger.

When the same atrial cells were raised to 35°C, this "notching" was no longer observable, probably due to the faster cellular repolarization at 35°C. Instead the cells developed spontaneous depolarizations between the stimulated beats. As the length of the perfusion with T_3 increased the frequency of the spontaneous beats also increased. When T_3 was removed from the perfusion solution, these spontaneous beats disappeared.

To investigated the mechanism responsible for the development of the spontaneous beats, ryanodine and thapsigargin were perfused along with T_3. Ryanodine and thapsigargin are both pharmacological compounds which inhibit the SR from accumulating Ca^{2+}, thereby preventing SR Ca^{2+} load (Janczewski & Lakatta, 1993; Rousseau *et al.*, 1987). Ryanodine (1 μM) and thapsigargin (5 μM), when perfused with T_3 in cells exhibiting spontaneous beats with T_3 alone, eliminated the arrhythmogenic effects of T_3. Therefore, these results imply that the spontaneous beats associated with T_3 application resulted from an increase in SR Ca^{2+} load. Kass *et al.* (1977) detailed evidence consistent with the idea that an oscillatory release of Ca^{2+} from internal stores is the primary event underlying development of aftercontractions (i.e. early after depolarization and delayed after depolarizations). As the SR gradually increased its Ca^{2+} load with T_3 application, occasional spontaneous releases of Ca^{2+} from the SR occurred leading at first to spontaneous subthreshold contractions, eventually leading to the generation of full spontaneous action potentials. Upon washout of T_3, as the SR Ca^{2+} load returned to normal levels, these spontaneous contractions subsided.

Finally the contractile behavior of isolated atrial cells were investigated. Contractility was quantified by measuring cell shortening in response to a stimulus sufficient to elicit an action potential. Upon the addition of T_3, within 15 minutes contractility increase 34.8 ± 1.6 %. Similar increases in tension/contractile development have been seen with acute T_3 exposure in experiments ranging from isolated muscle cells (Snow *et al.*, 1992, Mager *et al.*, 1992), to the isolated perfused heart (Tielens *et al.*,

1996), to the intact animal (Gøtzsche, 1994). Contractility is believed to increase with increased total cytosolic [Ca^{2+}] (Bers, 1991). Therefore, if more Ca^{2+} is released from the SR during each stimulus due to a greater Ca^{2+} load, a larger contraction will develop.

SUMMARY

These results support clinical findings seen in chronically hyperthyroid patients who traditionally present themselves with classic symptoms of increased heart rate, stroke volume, cardiac output and cardiac contractility along with potential atrial irregularities including atrial fibrillation (Klein & Ojamaa, 1994; Ridgeway, 1994; Natazawa *et al.*, 1994). The present experiments suggest the possible cellular mechanism responsible for the development of some of these symptoms originates from an increased Na^+ influx through the TTX-sensitive "fast" Na^+ channel. These results demonstrate that T_3 increases intracellular Na^+ concentration by increasing influx through the TTX-sensitive "fast" Na^+ channels. The L-type Ca^{2+} current was not shown to increase in response to an acute exposure of T_3. In previous work, T_3 was shown to increase the Na/Ca exchange current ($I_{Na/Ca}$) (Wang & Lipsius unpublished result). Therefore the rise in intracellular Na^+, through the TTX-sensitive Na^+ channel, is believed to stimulate Na/Ca exchange to raise intracellular Ca^{2+} concentration. This elevation in intracellular Ca^{2+} loads the sarcoplasmic reticulum (SR) with Ca^{2+}. With this augmentation in cellular calcium load, contractility increases as more calcium is released from internal stores during each stimulated beat. Overload of the SR with Ca^{2+} has been shown to lead to the development of Ca^{2+}-mediated delayed after depolarizations and spontaneous activity (Kass *et al.*,

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